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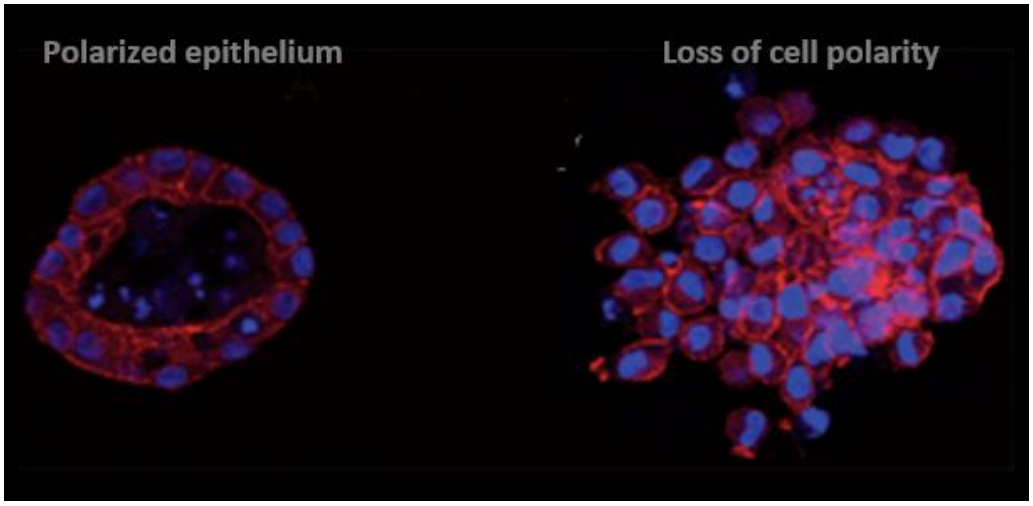
UNIVERSITY OF CRETE, DEPARTMENT OF BIOLOGY

INSTITUTE OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY - FORTH

Interplay between the cell polarity protein Scrib and signaling proteins in breast cancer.



Doctor of Philosophy

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Περίληψη

Η κυτταρική πολικότητα είναι καθοριστικής σημασίας για την αρχιτεκτονική και την λειτουργία όλων των ιστών του σώματος καθώς χαρακτηρίζει κυρίαρχες κυτταρικές λειτουργίες όπως η ασύμμετρη κυτταρική διαίρεση και η κυτταρική μετανάστευση. Τα φυσιολογικά επιθηλιακά κύτταρα είναι το πιο χαρακτηριστικό παράδειγμα κυττάρων που εμφανίζουν κυτταρική πολικότητα (κορυφαία – βασική πολικότητα) με την κορυφαία επιφάνεια να εκτίθεται στον αυλό και την βασική επιφάνεια να βρίσκεται προσκολλημένη στη βασική μεμβράνη. Για την εγκαθίδρυση και την διατήρηση της κυτταρικής πολικότητας είναι υπεύθυνα τρία κύρια πρωτεϊνικά σχήματα, τα Crumbs (Crumbs3-PALS1-PATJ) και Par (Par3-Par6-aPKC) σχήματα προσδιορίζουν την κορυφαία επιφάνεια του κυττάρου, ενώ το Scribble σχήμα (Lgl-Scrib-Dlg) προσδιορίζει την πλαγιο-βασική επιφάνεια του κυττάρου. Στα επιθηλιακά κύτταρα, οι πρωτεΐνες κυτταρικής πολικότητας στρατολογούνται στις διακυτταρικές συνδέσεις όπου αλληλεπιδρούν με πρωτεΐνες προσκόλλησης καθώς και με άλλες πρωτεΐνες, συστατικά σηματοδοτικών μονοπατιών που ρυθμίζουν την κυτταρική αύξηση, την διαφοροποίηση, την απόπτωση και την επιθηλιακή-μεσεγχυματική μετάπτωση (EMT).

Αλλαγές στην δομή ή στη ρύθμιση του Scrib γονιδίου που οδηγούν σε μη φυσιολογικά πρωτεϊνικά επίπεδα ή σε εσφαλμένο κυτταρικό εντοπισμό εξαιτίας διαφόρων σημειακών μεταλλαγών ή γονιδιακής ενίσχυσης έχουν παρατηρηθεί σε ποικίλες περιπτώσεις καρκίνου. Διάφορες μελέτες έχουν δείξει ότι η Scrib πρωτεΐνη δρα συνεργιστικά με ενεργοποιημένα ογκογόνα σηματοδοτικά μονοπάτια, όπως τα Ras, ErbB2 και TGFβ σηματοδοτικά μονοπάτια, με αποτέλεσμα την προαγωγή του σχηματισμού του όγκου και την μετάσταση. Αυτή η μελέτη αποκαλύπτει τον ρόλο μίας κολοβωμένης μορφής της Scrib πρωτεΐνης στη ρύθμιση των mTOR, Wnt και JNK σηματοδοτικών μονοπατιών σε ανθρώπινες κυτταρικές σειρές καρκίνου του μαστού. Συνολικά, αυτή η μελέτη αποδεικνύει ότι η κολοβωμένη πρωτεΐνη Scrib94-494 λειτουργεί ως ένας κύριος ρυθμιστής σηματοδοτικών γεγονότων που κατέχουν κυρίαρχο ρόλο στην ρύθμιση του αποπτωτικού κυτταρικού θανάτου και της κυτταρικής διείσδυσης.

Abstract

Cell polarity is fundamental for the architecture and function of all body tissues by determining crucial cellular functions such as asymmetric cell division and cell migration. Normal epithelial cells are a typical example of polarized cells (apico-basal polarity) with the apical surface exposed to the lumen and the basal surface attached to the basement membrane. Three main protein complexes are responsible for the establishment of cell polarity, the Crumbs (Crumbs3-PALS1-PATJ) and Par (Par3-Par6-aPKC) complexes identify the apical domain of the cell, while the Scribble complex [lethal giant larvae (Lgl) - Scribble (Scrib) - Disc large (Dlg)] defines the basal domain. In epithelial cells, polarity proteins are recruited at intercellular connections where they associate with adhesion proteins as well as other proteins, components of signaling pathways that regulate cell growth, differentiation, apoptosis and epithelial-mesenchymal transition (EMT).

Alterations of Scrib polarity gene structure or regulation that leads to abnormal protein levels or subcellular localization due to various point mutations or gene amplification have been observed in several cases of cancer. Scrib has been found to act synergistically with activated oncogenic signaling pathways such as Ras, ErbB2 and TGFβ to promote tumor formation and metastasis. This study unveils a role for a truncated form of Scrib that has been found in several cancers, in regulating mTOR, Wnt and JNK signaling in human breast cancer cell lines. Overall, this study defines the truncated Scrib94-494 as a major regulator of signaling events with executive role in apoptotic cell death and cell invasion.

1. Introduction

1.1 Cell polarity

At the cellular level, symmetry-breaking manifests as cell polarity. Cell polarity refers to the asymmetric distribution of several cellular components, such as organelles, cytoskeleton, macromolecules and metabolites, building distinct subcellular domains with specific functions. In multicellular organisms, almost all cell types exhibit some kind of polarity which is fundamental for the architecture and function of all body tissues by determining crucial cellular functions (Figure 1.1.i).

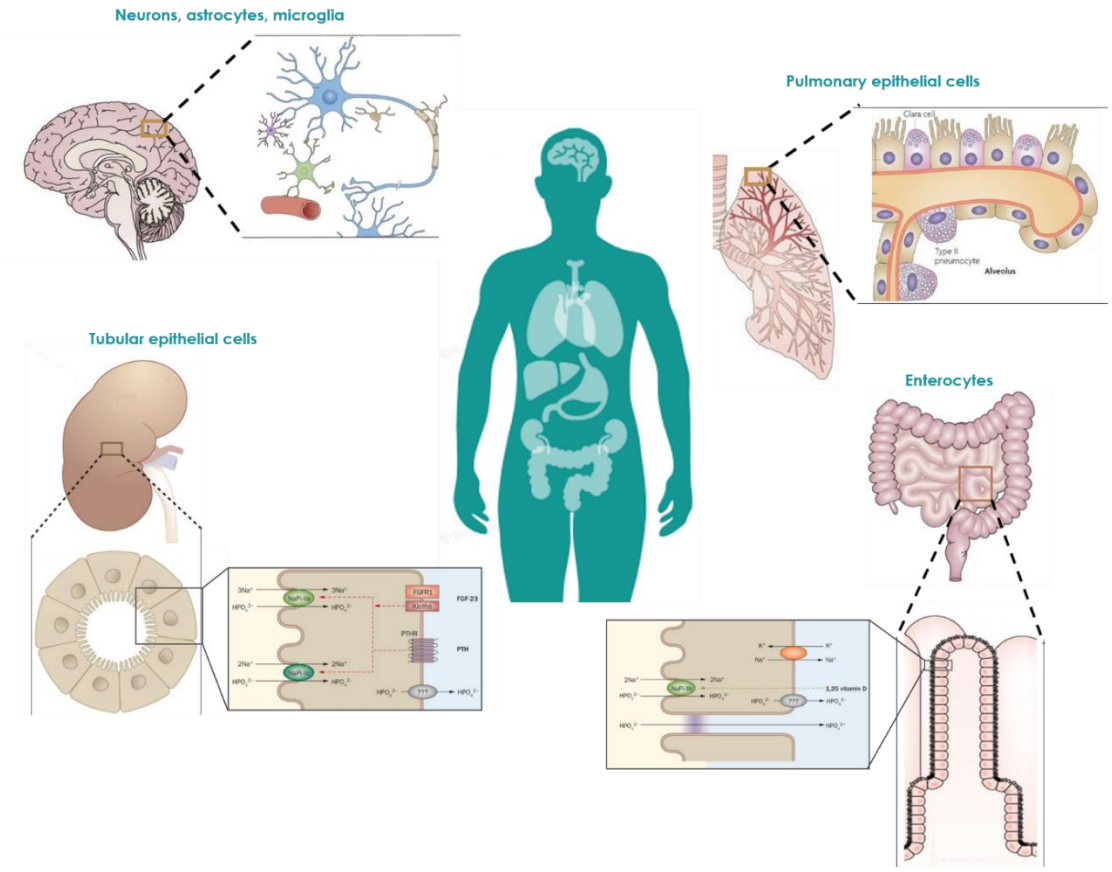


Figure 1.1.i
Characteristic examples of cell polarization in human body.

Cell polarity localizes different activities to distinct regions of cells and it is central for the maintenance of tight cell–cell contacts and normal tissue integrity as well as cell

migration and development. *C.elegans* and *Drosophila* developmental genetics, along with mammalian epithelial cell biology, have identified conserved proteins and mechanisms that are responsible for the establishment of cell polarity. Extracellular diffusible molecules and their surface receptors, interactions with the extracellular matrix and with other cells, transmembrane proteins, polarity determinants, scaffold proteins, signaling proteins, cytoskeletal structures and protein traffic are the major players in establishing cell polarity.

1.1.1 Apical-basal polarity

Normal epithelial cells are a typical example of polarized cells that display apico-basal polarity with the apical surface exposed to the lumen and the basal surface attached to the basement membrane, serving essential functions, such as tissue morphogenesis, tissue protection, selective absorption and secretion. The polarized morphology of the cell, reflects the polarization of the intracellular organization and signaling cascades. Three main protein modules that function from spatially restricted domains, are responsible for the establishment of cell polarity, the Crumbs (Crumbs3-PALS1-PATJ) and Par (Par3-Par6-aPKC-Cdc42) modules identify the apical domain of the cell, while the Scribble module [lethal giant larvae (Lgl) - Scribble (Scrib) - Disc large (Dlg)] defines the basal domain (Figure 1.1.1.i). In epithelial cells, polarity proteins are recruited at intercellular connections where they associate with adhesion proteins (Yates et al. 2013) as well as other proteins, components of signaling pathways that regulate cell growth, differentiation, apoptosis and epithelial-mesenchymal transition (EMT).

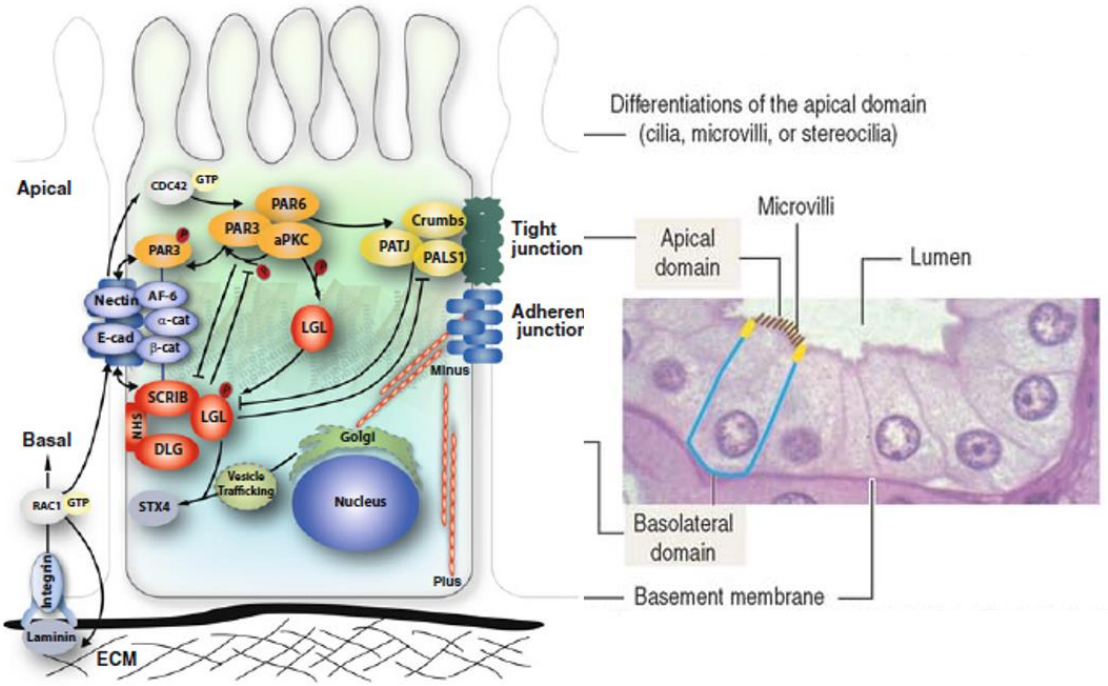


Figure 1.1.1.i
Protein modules in epithelial apico-basal polarity.

Epithelial cells display polarized distribution of most membrane proteins, such as ion channels, transporters, pumps and growth factor receptors. The generation of cell surface polarity derives from sorting signals encoded in the amino acid sequence of cell surface proteins, as well as the interplay of polarity modules with trafficking routes that include apical or basolateral recycling endosomes and with epithelial-specific protein complexes such as Adaptor Protein (AP) complexes and clathrin, which can be regulated by small GTPases. The Na+ - K+ ATPase found in the plasma membrane of all animal cells, is assembled in the endoplasmic reticulum, sorted in the trans-Golgi network, and delivered to the basolateral membrane of numerous epithelial cells. In renal epithelium, proximal tubular epithelial cells HCO3– reabsorption depends on the action of the apical distributed sodium hydrogen exchanger NHE3 as well as cotransporter NBCn2 (Guo et al., 2017).

Apico-basal polarity function in development and epithelial morphogenesis by maintaining overall tissue integrity while individual cells detach from epithelial sheets. Furthermore, in several cases tissue-intrinsic apico-basal polarity is inherited to delaminated cells during embryogenesis; for instance, in *D. melanogaster* embryos, the

neural stem cells called neuroblasts delaminate from an apical-basal polarized neuroectoderm, maintaining the intrinsic apical-basal polarity axis in relation to the overlying neuroepithelium (Homem and Knoblich 2012). Figure 1.1.1.ii

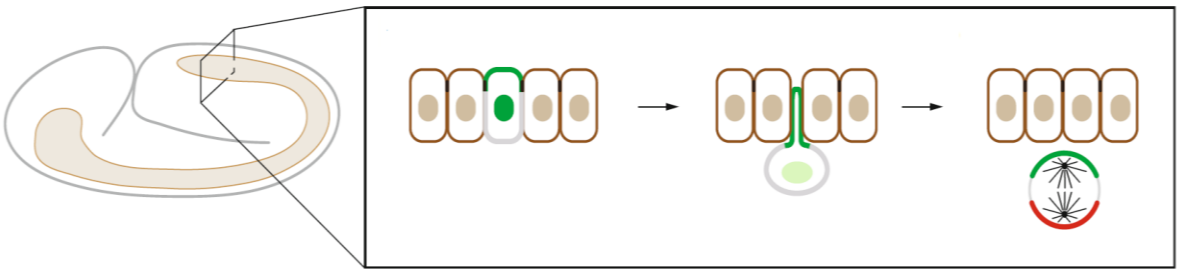


Figure 1.1.1.ii
Neuroblast delamination during embryonic development.

Additionally, polarity proteins play a key role in epithelial differentiation, as elevated levels of the cell polarity proteins Scrib and Dlg and changes in protein distribution have been reported during epithelial cell differentiation. In the lower part of the colon crypt where reside highly proliferating cells, Scrib and Dlg proteins were expressed at low levels with a typical basolateral distribution. During cell migration from the bottom of the crypt toward the upper in contact with the intestinal lumen part where differentiated cells reside, there is a gradual increase in the levels of Scrib and Dlg, and alterations in the cellular localization pattern (Gardiol et al., 2006).

1.1.2 Asymmetric cell divisions

The development of multicellular organisms implicates the specification of various cell types from a single fertilized egg. To generate this diversity, some cells undergo asymmetric cell division, during which they differentially segregate polarity determinants into the two daughter cells, thereby determining distinct cell fates (Figure 1.1.2.i). In both embryonic and adult state, the portioning of cellular components in response to polarizing cues during mitosis is important for development as well as tissue homeostasis and differentiation. Asymmetric cell division generates a committed

progenitor daughter cell and a noncommitted daughter stem cell. Cell polarity is the central organizer of asymmetric cell division, guiding the segregation of cell fate determinants and spindle orientation positioning.

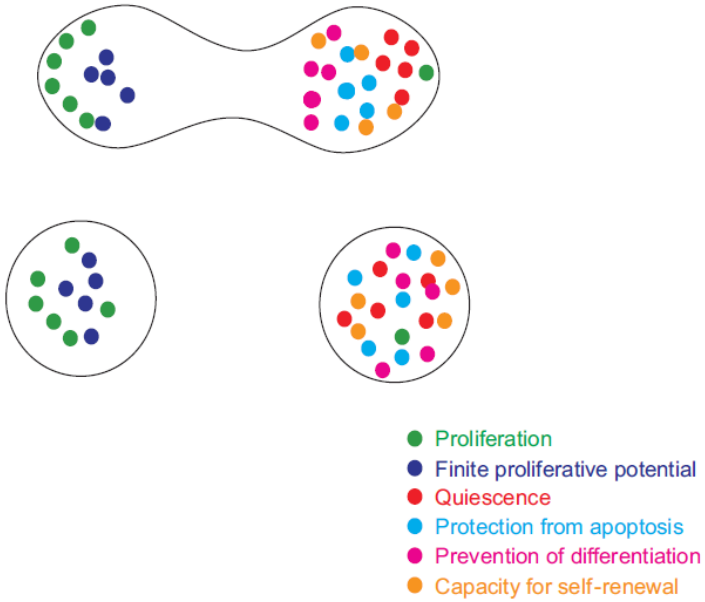


Figure 1.1.2.i

Asymmetric cell division involves asymmetric distribution of multiple cell fate determinants into each of the daughter cells.

Irrespective of context and purpose, asymmetric cell division requires accurate spatiotemporal coordination of the following processes (Figure 1.1.2.ii): 1) establishment of an intrinsic polarity axis, distributing polarity proteins to specific subcellular domains, 2) orientation of the mitotic spindle and chromosome segregation, 3) asymmetric localization of cell fate determinants and 4) site specification of cytokinetic furrow resulting in biased segregation of both apical and basal proteins between sibling cells (Homem and Knoblich 2012, Li et al., 2013).

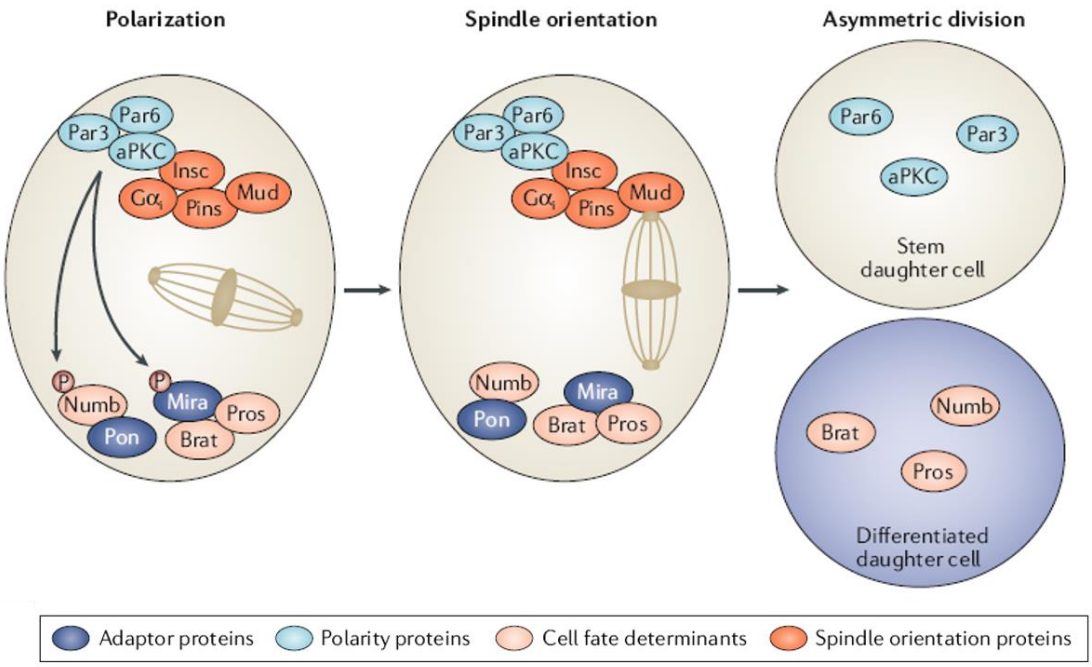


Figure 1.1.2.ii

The sequence of molecular events leading to an asymmetric cell division. From Lytle et al., 2018

Polarity establishment and spindle orientation is orchestrated in a sequence in which when the cell enters anaphase, cell fate determinants are correctly distributed between the two sibling cells. The spindle has a key role, since its orientation determines the axis of cell division and thereby decides about symmetry or asymmetry of the division. (Figure 1.1.2.iii)

1.1.2.1 Model systems for asymmetric cell divisions

Caenorhabditis elegans zygote, *Drosophila melanogaster* neuroblasts as well as mammalian hematopoietic cells have been useful model systems for studying the molecular mechanisms of asymmetric cell division.

1. Asymmetric cell division in *C. elegans*:

Asymmetric cell division is a recurrent process in the generation of diverse cell lineages during *C. elegans* embryonic development, starting at the first zygotic division. In *C.*

elegans zygote, PAR-3, PAR-6 and aPKC accumulate anterioriorly, while PAR-1 and PAR-2 accumulate posteriorly. (Figure 1.1.2.iv)

2. Asymmetric cell division in *D. melanogaster*:

During embryogenesis in *D. melanogaster* embryos, the neural stem cells called neuroblasts are subjected to epithelial-to-mesenchymal transition (EMT), subsequently they delaminate from the apical-basal polarized neuroectoderm and they enter mitosis undergoing asymmetric cell divisions. Asymmetric cell division of a neuroblasts generates an apical self-renewed neuroblast and a smaller basal differentiating ganglion mother cell (GMC) that divides to two daughters which are usually neurons but in some cases can be glia. The intrinsic polarity axis of *Drosophila* neuroblasts is most likely inherited during embryonic delamination from the polarized neuroectoderm (Homem and Knoblich 2012). Embryonic neuroblasts can undergo a number of asymmetric divisions, maintaining the intrinsic polarity axis in relation to the overlying neuroepithelium. (Figure 1.1.2.iv)

3. Asymmetric cell division in hematopoietic cells:

A characteristic example of asymmetric division in hematopoietic cells is erythroblast enucleation, during which an interphase cell ejects its nucleus, giving rise to an anucleate reticulocyte (Li, 2013) (Figure 1.1.2.iv). Hematopoietic multipotent progenitor cells (MPP) divide asymmetrically, giving rise to lymphomyeloid and erythromyeloid lineages (Görgens et al., 2014). Additionally, T lymphocytes, upon activation by antigen-presenting cells APCs, undergo asymmetric cell division. The daughter cell proximal to the APC more likely differentiate into an effector-like T cell whereas the distal presumably differentiate into a memory-like T cell (Figure 1.1.2.v). In a recent study, it has been shown that c-Myc is segregated with Numb and Scrib in proximal daughters. Furthermore, they noticed that the asymmetric distribution of c-Myc comes along with the asymmetric distribution of amino acid transporters, amino acid content, and activity of mammalian target of rapamycin complex 1 (mTORC1). Cell polarity regulates many physiologically processes in leukocytes, such as migration, secretion of cytolytic granules and cytokines, and compartmentalization of signaling (Zigmond et al., 1981, Russell, 2008).

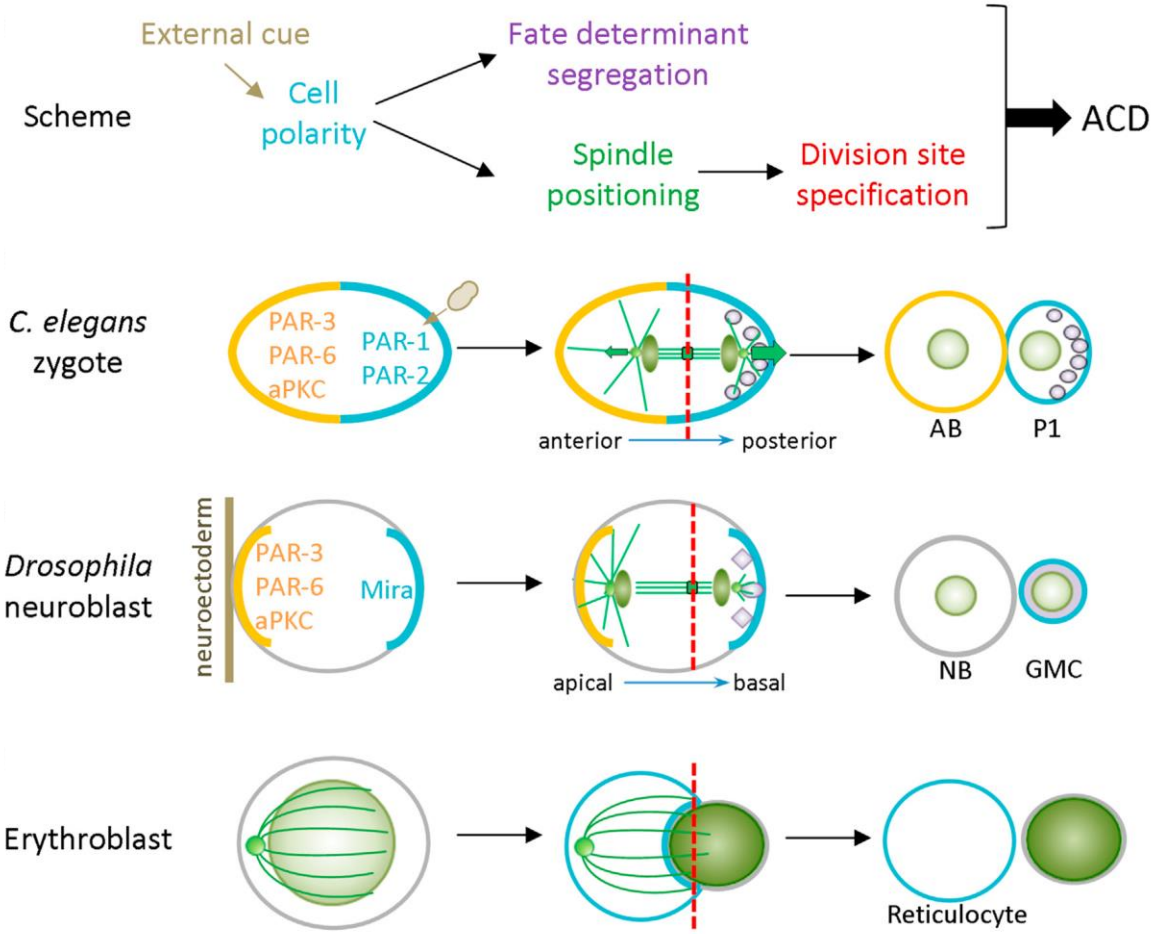


Figure 1.1.2.iv

The common scheme of asymmetric cell division shared in mitotic animal cells and erythroblast enucleation. The spindle midzone complex determines the site of cell division. From Li, 2013

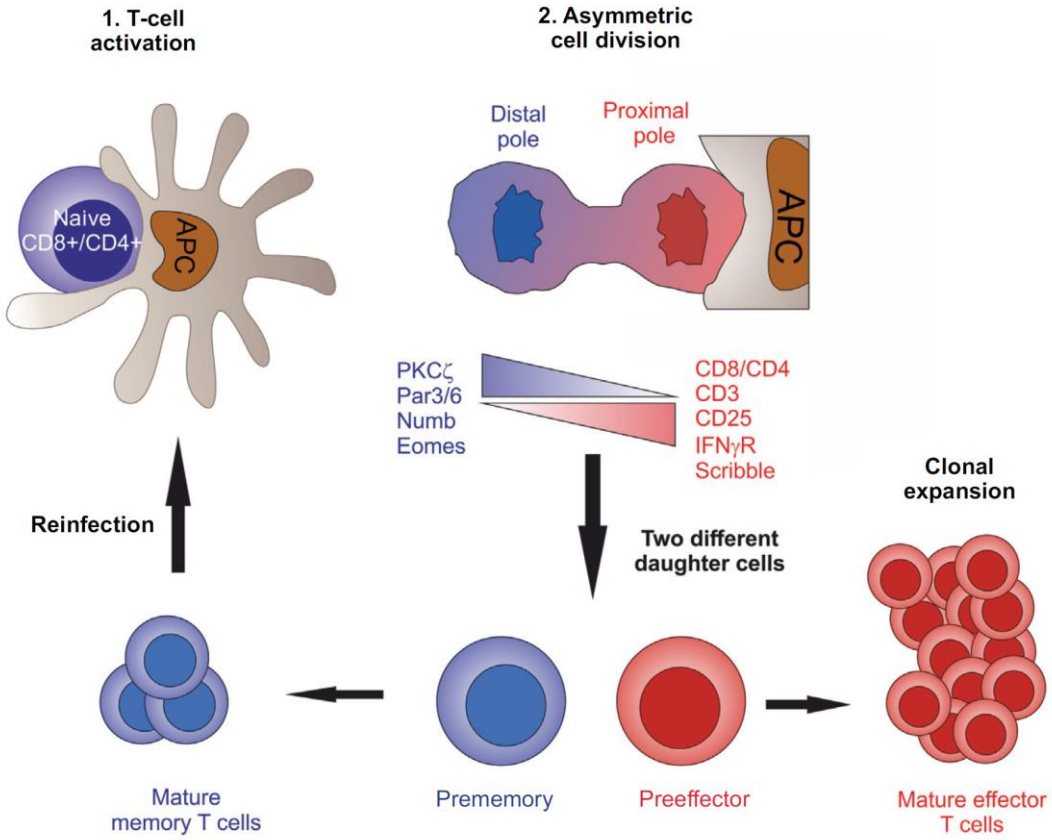
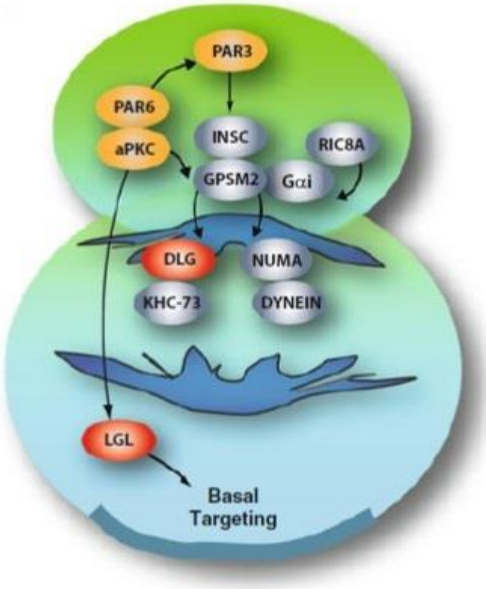


Figure 1.1.2.v
Asymmetric cell division upon T-cell activation. From Bustos-Morán et al. 2016

Mechanistically, on entry into mitosis during asymmetric cell division AuroraA phosphorylates Partitioning defective 6 (Par6) which in turn activates atypical protein kinase C (aPKC), leading to Lethal giant larvae (Lgl) phosphorylation and the subsequent exchange of Lgl for Par3 into the aPKC-Par6 complex. Par3 is now localized at the apical cell cortex where it interacts with Inscuteable (Insc) which in turn recruits the molecular link LGN. Then, the apical LGN functions via two parallel pathways the Dlg-KHC-73 and the NuMA-Gαi-Dynein pathways, to both anchor astral microtubules and generate pulling forces required for cytokinesis, respectively. Phosphorylation of Par-6 via AurA leads to the activation of aPKC which in turn phosphorylates Lgl is required for appropriate localization of basal cell fate determinants. (Figure 1.1.2.vi)

Figure 1.1.2.vi

The core polarity modules during asymmetric cell division.



1.1.2.2 Cell fate determinants

The *D. melanogaster* homologues of the anterior Par proteins direct the asymmetric localization of a cell fate determinant, called Numb into one of the two daughter cells. In 1994, the *Drosophila* Numb was found to localize to one edge of the cell during mitosis, forming a crescent-shaped pattern, and to segregate into only one of the two daughter cells (Rhyu et al., 1994). The cell fate determinant Numb antagonizes the activity of Notch signaling during asymmetric cell division in development (Guo et al., 1996). In the absence of Numb, normally different cells appear the same fate in *D. melanogaster* external sensory organs (Uemura et al., 1989). The endocytic protein Numb and the translation inhibitor Brain tumour (BRAT), accumulate at the basal plasma membrane in late prometaphase during asymmetric cell division of Drosophila neuroblasts. Their asymmetric localization is facilitated by the adaptor proteins Partner of Numb (PON) and Miranda respectively. Slightly after the basal determinants localize, the mitotic spindle is set up in an apical–basal orientation so that these determinants are inherited by the basal daughter cell, providing differentiation properties (Figure 1.1.2.1.i). Additionally, when bound to Par3, aPKC phosphorylates Numb, regulating its segregation into the basal daughter cell.

(Knoblich, 2010) It is remarkable that the cell fate determinant Numb is considered to selectively binds to certain transmembrane receptors and links them to the endocytic machinery for internalization of receptors (Sorkin 2004).

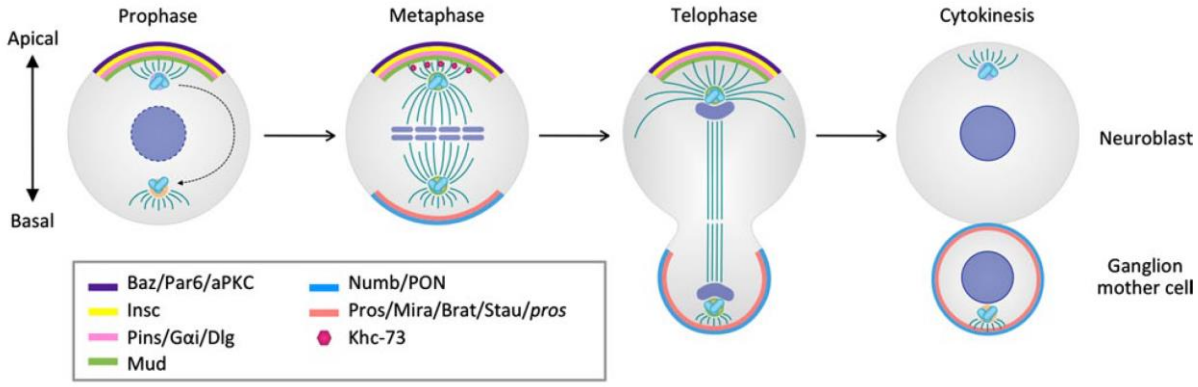


Figure 1.1.2.1.i

Asymmetric segregation of cell polarity and cell fate determinants at different stages of mitosis in neuroblasts. From Gomez-Lopez et al. 2014

1.1.3 Front-rear cell polarity

Cell migration is a cellular function that involves cell polarization and function during development as well as in the adult in physiology and disease where it is crucial for immune reaction, wound healing, tissue renewal and cancer spreading respectively. Migrating cells display a front-rear polarity, with a protruding front and a retracting trailing edge (Figure 1.1.3.i). Random migration requires the establishment of a front-rear polarity axis, while the directed migration requires the specific orientation and the maintenance over time of the front-rear polarity axis in a direction provided by several cues of the cell environment. Migration occurs either in individual cells or in group of cells (collective migration) and usually within a dense environment composed of extracellular matrix and other cells. Major players in cell migration are the cytoskeleton rearrangements as well as the vesicular traffic that controls the directed delivery of membrane components (Figure 1.1.3.ii).

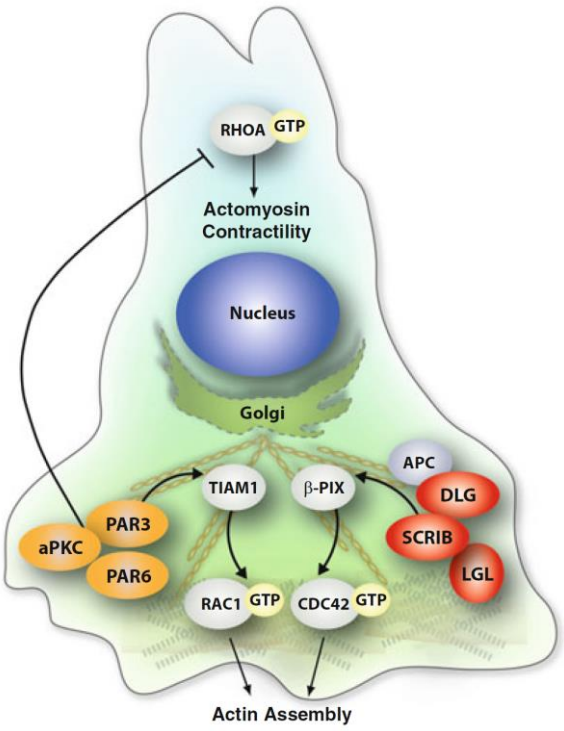


Figure 1.1.3.i

Scrib and Par polarity modules in front-rear polarity of a migratory cell.

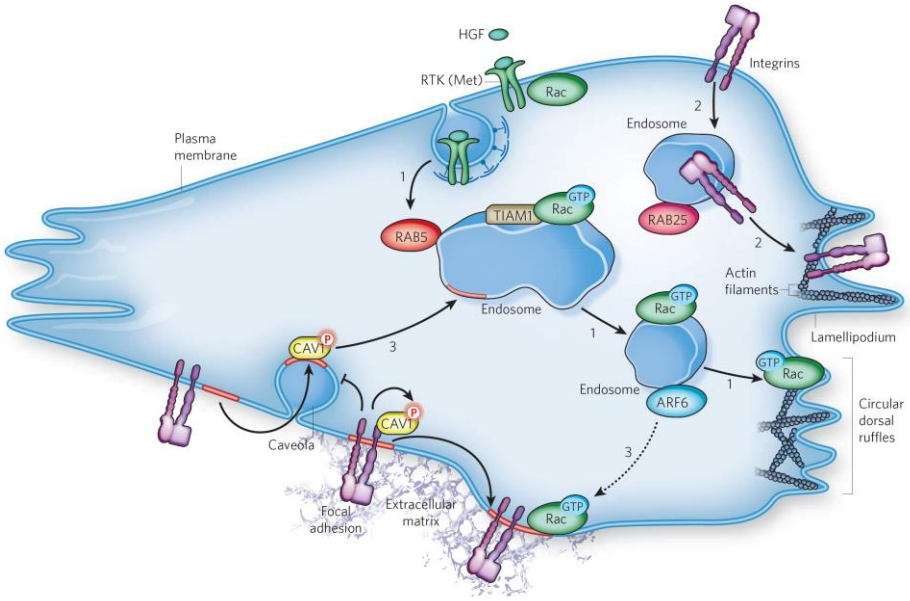


Figure 1.1.3.ii
Vesicular trafficking and membrane protrusions such as ruffles and invadopodia, in migratory cells. From Scita and Di Fiore, 2010

The organization of the cell machinery along a front-rear axis is mandatory for cell migration. For instance, during epithelial cell migration the nucleus is typically positioned in the rear part of the cell, which it serves as an indicator of the cell polarity axis (Dupin et al., 2009), whereas at the front edge of the cell are formed adhesive contacts and cell protrusions like fillopodia, lamellipodia and invadopodia. Activated and migrating T cells extend a single protrusion called uropod, which contains adhesion receptors for interaction with other lymphocytes (Gerard et al., 2007). During directed migration of endothelial cells, a single protrusion lamellipodium is formed at their leading front, which is characterized by Rac1 accumulation. Silencing of the polarity protein Scrib increases the mean number of lamellipodia per endothelial cell and the orientation of these protrusions toward a chemotactic gradient is lost. Scrib regulates endothelial cell polarization and orientation during directed migration via its PDZ and LRR domain, controlling Rac localization and integrin α5 cell surface expression (Michaelis et al., 2013).

1.1.3.1 Migration and invasion of migratory cells

Migratory cells remodel the nearby tissue environment by opening passageways through the extracellular matrix (ECM). To do so, migratory cells secrete proteases, such as matrix metalloproteinases (MMPs), dissolving the dense ECM molecules that surround the cells within tissues. The ECM components that are cleaved by MMPs are fibronectin, tenascin, laminin, collagens and proteoglycans. MT1-MMP (membrane type 1- MMP) is a type of membrane-anchored metalloproteinase and therefore they are able to cleave substrate proteins in the close vicinity of the cells that produce them. Additionally, MT1-MMP may attack and cleave cell surface adhesion molecules, such as cadherins and integrins, as well as growth factor receptors and chemokines. It can also cleave inactive pro-enzymes, such as pro-MMP-2, into enzymatically active MMPs.

It is proven that specific protrusions called invadopodia are formed in migratory human cancer cells. At the first stages of invadopodium formation, cells establish focal

adhesions with the extracellular matrix (ECM) through the interaction of integrins, SRC and Tyr397-phosphorylated focal adhesion kinase (FAK). Matured invadopodia promote the degradation of the ECM by the secretion of matrix metalloproteases (MMP2, MMP9), as well as by the delivery and membrane presentation of the membrane type 1 MMP (MT1MMP) to the tip of the protrusion, through the interaction of these proteases. Cortactin, Tyr kinase substrate with four SH3 domains (TKS4) and β1 integrin constitute key contents of matured invadopodia (Murphy et al., 2011).

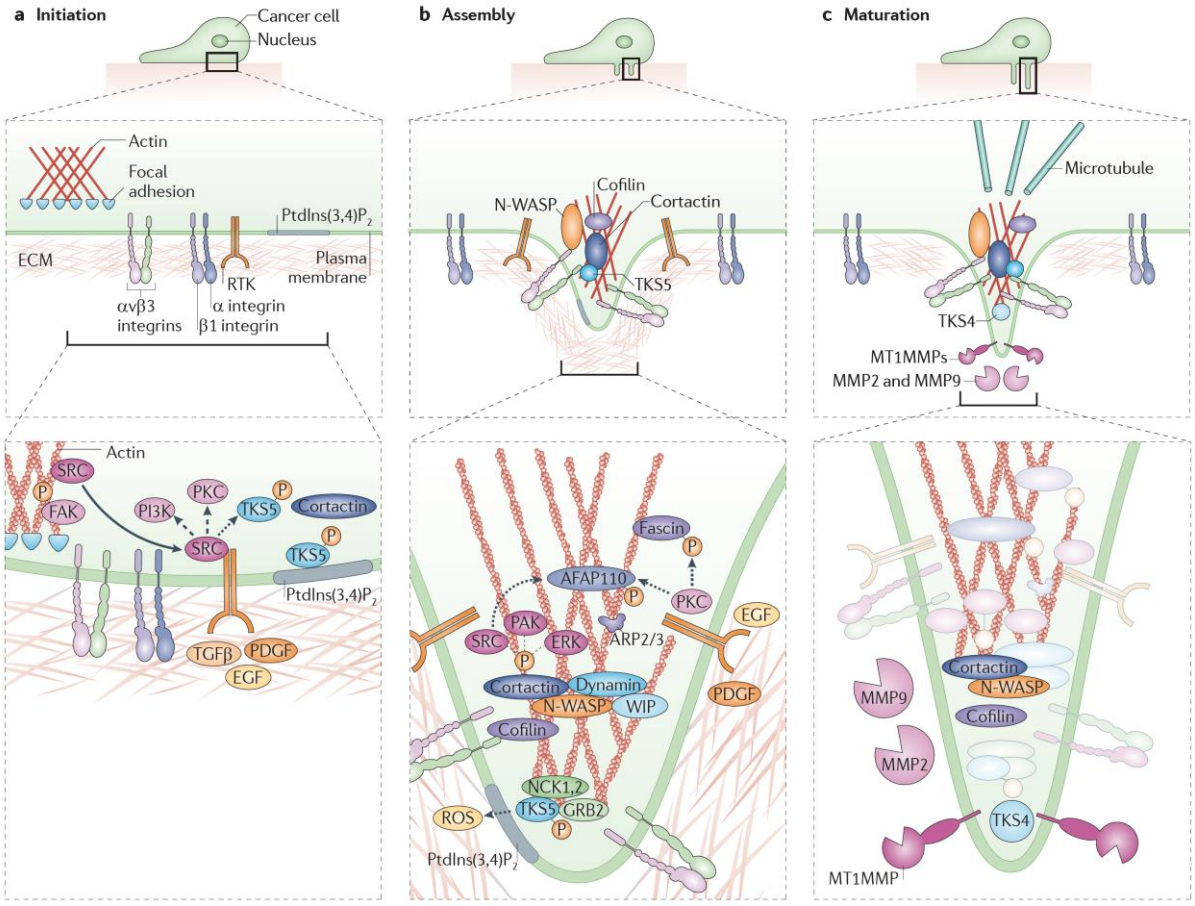


Figure 1.1.3.1.i
The stages of invadopodia formation. From Murphy et al., 2011

In contrast to the dot-shaped invadopodia, focal adhesions (FAs) are streak-like structures at the basal membrane that bridge the actin cytoskeleton to the ECM. Focal adhesions are multiple assemblies of proteins forming at the sites of clustered integrin adhesion and consisting of talin, vinculin, focal adhesion kinase (FAK), and paxillin that anchor bundles of actin stress fibers and transfer signals intracellularly. The assembly of focal adhesions at the cell front is driven by Rho GTPase and integrin engagement. The dynamic turnover of FAs is essential for the motility of both normal and tumor cells and it is mediated by recycling of integrins from the rear of a cell to its front via endocytosis. Despite distinct morphologies, FAs and invadopodia share many components, including integrins, talin, paxillin, actin, cortactin, and dynamin. It has been known that a variety of cancer cell lines degrade matrix not only at invadopodia, but also at numerous peripheral sites that represent bona fide FA sites. This FA-type degradation is MT1-MMP dependent. Mechanistically, MT1-MMP is targeted to FAs by a physical interaction with a FAK–p130Cas complex in response to the activation of Src. Disrupting the FAK–p130Cas–MT1 complex significantly impairs FA-mediated degradation and tumor cell invasion yet does not appear to affect invadopodia formation or function (Wang et al., 2012).

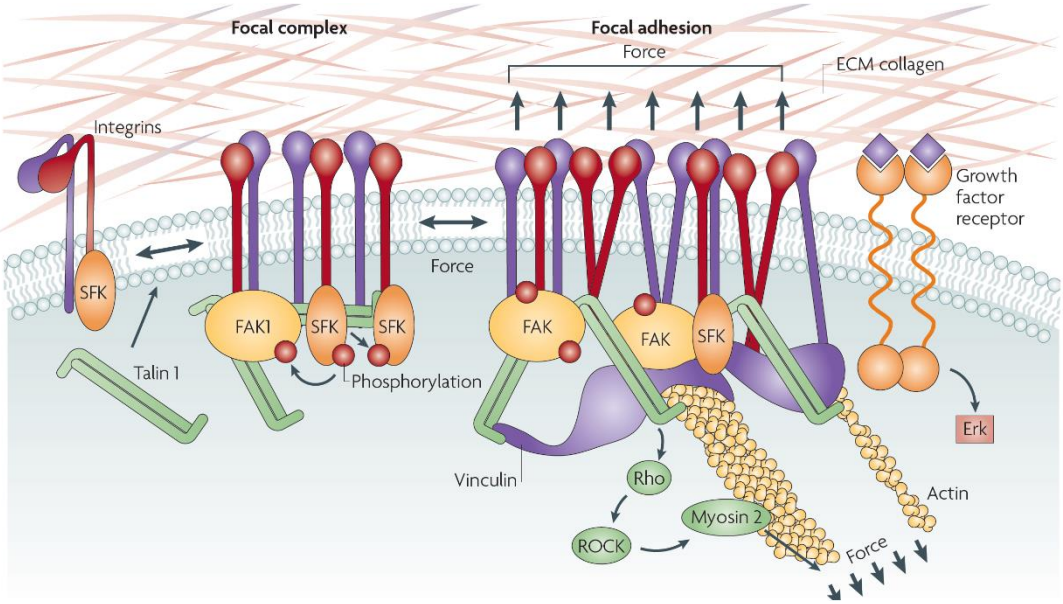


Figure 1.1.3.1.ii
Focal adhesion maturation. From Butcher et al., 2009

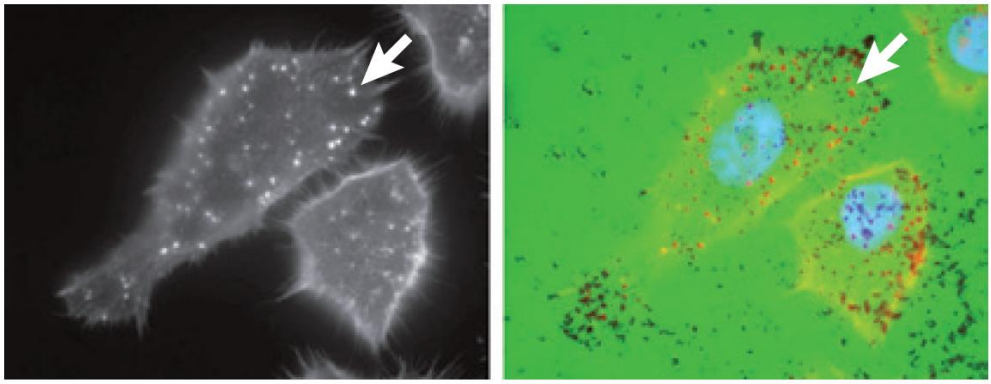


Figure 1.1.3.1.iii
Dot-shaped matrix degradation pattern of invadopodia. Cells plated on fluorescent gelatin (green)-coated coverslips. From Murphy et al., 2011

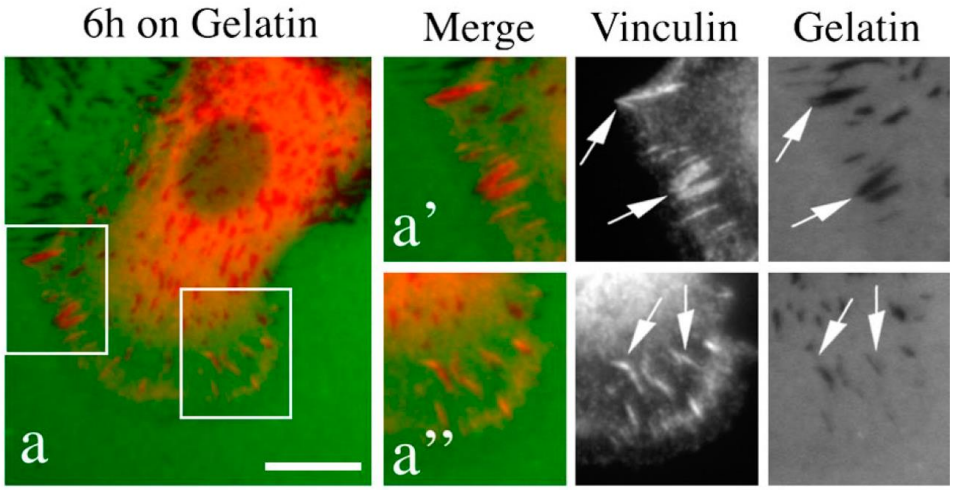


Figure 1.1.3.1.iv
Matrix degradation at vinculin-positive Focal Adhesion sites. Cells plated on fluorescent gelatin (green)-coated coverslips. From Wang et al., 2012

1.1.4 Planar cell polarity

At a tissue level, cell polarity refers to three-dimensional structures that are sustained by intracellular and intercellular interactions and are characterized by organized arrangement of cells in geometrically specified body axes (Figure 1.1.4.i). Planar cell polarity describes the alignment of cells within the plane of a tissue that is perpendicular to the apicobasal axis and a prerequisite for oriented cell division. In epithelia, planar orientation of divisions is required for the maintenance of daughter

cells in the plane of the tissue. Additionally, proper epithelial cell arrangements and cell-cell contacts are critical for the formation of a network of tubes in several organs. Breast ductal epithelium and renal tubular epithelium are characteristic examples of epithelia that form a central lumen and defined polarity domains. In this context, during mitosis, spindle orientation occurs in the plane of the epithelium and depends on the lateral localization of molecular adaptors able to anchor astral microtubules to the lateral cell cortex (Figure 1.1.4.ii). Daughter cells resulting from a division must be correctly positioned in order to maintain tissue structure. Evidence that Scrib regulates planar cell polarity of epithelial cells has been supported by the fact that the lack of Scrib causes a defect in the planar polarization of the mouse inner ear (Montcouquiol et al., 2003). Since, Scrib plays a key role in the assembly and maintenance of junctional complexes, adherens and tight junctions, its role is crucial for proper tissue organization.

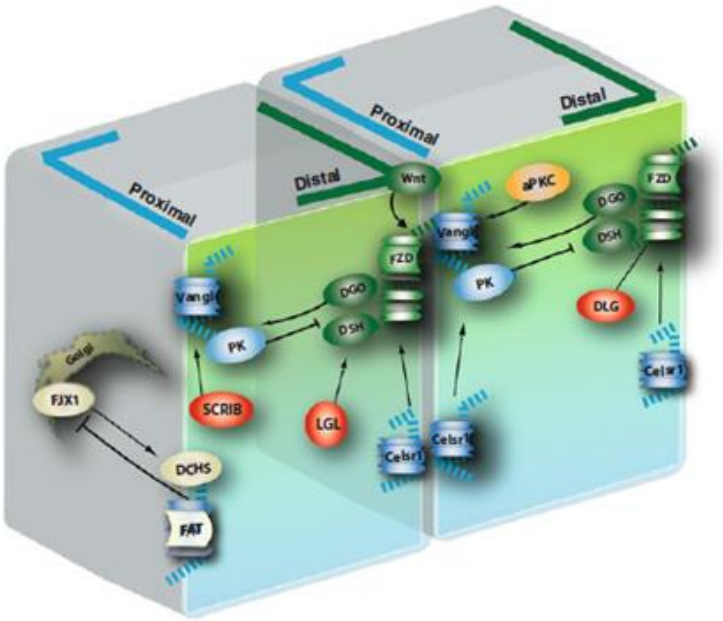


Figure 1.1.4.i

Protein modules in planar cell polarity.

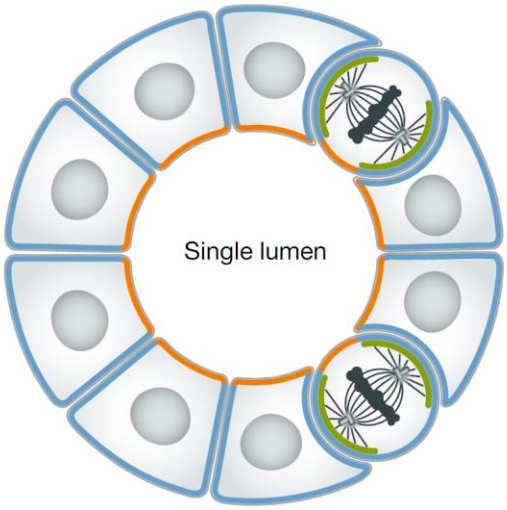


Figure 1.1.4.ii

Planar polarity of an epithelial lumen.

1.1.5 Neuronal Polarity

Neurons are polarized cells that have multiple neurites, a single axon and numerous dendrites. Axons and dendrites contain specific cytosolic polarity factors, cytoskeletal elements as well as membrane proteins such as neurotransmitter receptors and ion channels. The Scrib polarity module has been shown to play a role in neuronal polarization and function. More specifically, Drosophila Scrib localizes to synapses in association with GUKh in a Dlg-dependent manner. Mouse Scrib has been proven to be enriched at synapses of primary hippocampal neurons, as well as in their “spine” precursors colocalized with the cadherin–catenin complex. Moreover, Scrib1 has been shown to be localized postsynaptically at NMDA excitatory synapses in the stratum radiatum in CA1 of the hippocampus as well as in intracellular vesicles or organelles in the dendritic spine, regulating NMDA recycling. Additionally, Scrib promotes axon myelination in postnatal oligodendrocytes. (Bonello and Peifer, 2018)

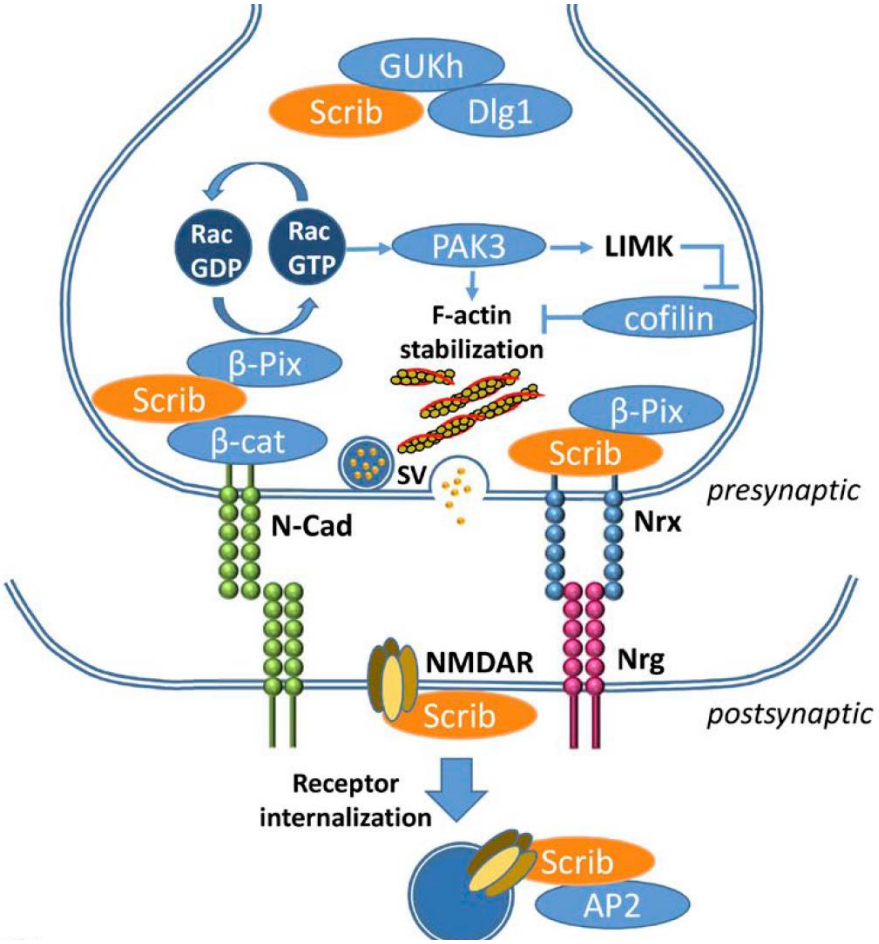


Figure 1.1.5.i

Scrib regulates neural function. From Bonello and Peifer 2018.

1.1.6 Immune synapse polarity

Different forms of polarity have been observed in lymphocytes. Migrating neutrophils and T cells have a leading edge rich in chemokine receptors. Additionally, lymphocytes polarize during activation by antigen-presenting cells to form an immunological synapse, leading to a signaling response. Both aPKC and Par3 polarize significantly to the distal side of the cell in early mitotic T cells, maintaining this asymmetry during late mitosis, whereas Scribble and DlgF significantly polarize to the proximal cell in early and late mitosis. The cell fate determinant Numb is polarized to the distal pole of mitotic cells, and this asymmetry is maintained through to cytokinesis (Oliaro et al., 2010).

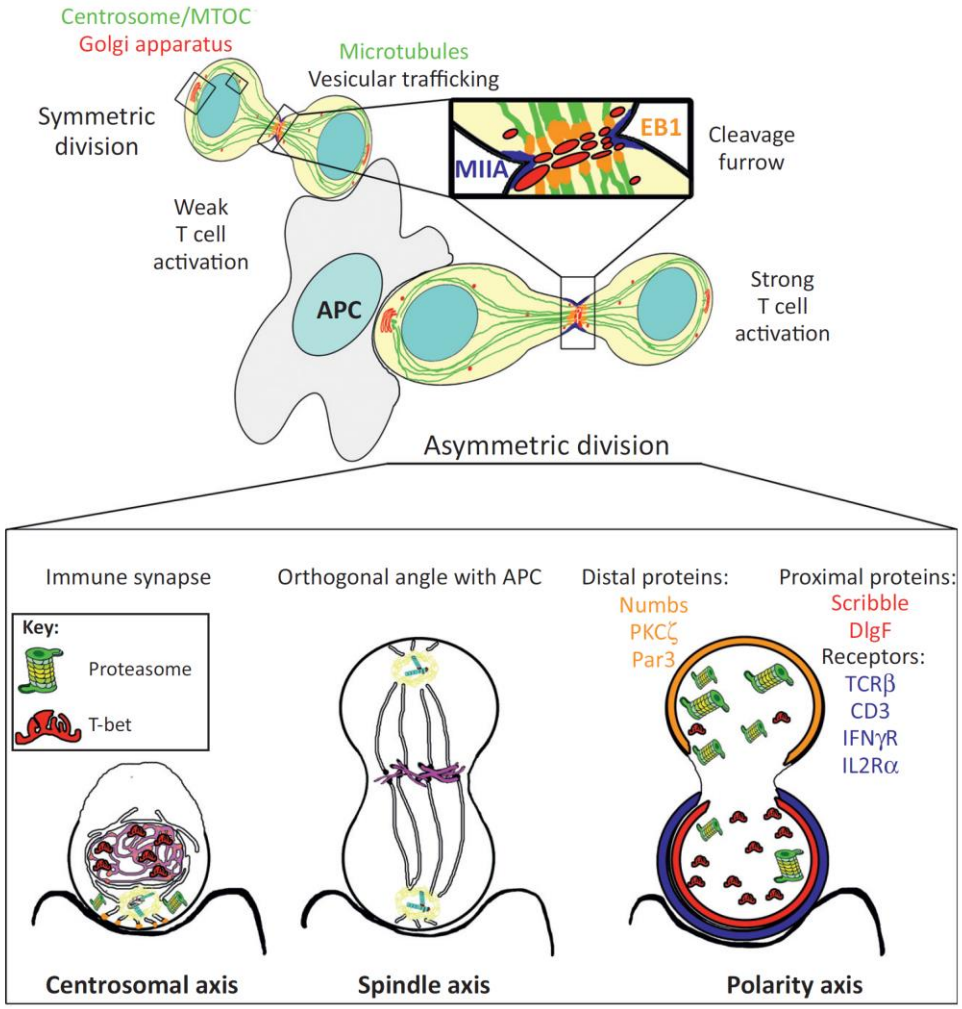


Figure 1.1.6.i

Asymmetric distribution of cell polarity proteins and cell fate determinants in immune synapse. From Martín-Cófreces et al. 2015.

1.1.7 Plasticity in cell polarity during fate transition processes

Epithelial-to-Mesenchymal Transition (EMT), Endothelial-to-Mesenchymal Transition (EndMT) as well as Endothelial-to-hematopoietic cell transition (EHT) are crucial developmental processes that are characterized by alterations in cell polarity orientation and destabilization of cell junctions. EMT and EndMT are highly dynamic reversible processes, whereas the opposite mechanism is termed Mesenchymal-to-Endothelial Transition (MET) and Mesenchymal-to-Endothelial Transition (MEnT) respectively. EMT is observed during gastrulation, embryogenesis and cancer. EMT

results in the transdifferentiation of epithelial cells into motile mesenchymal cells. This switch in cell differentiation and behaviour is mediated by key transcription factors, including SNAIL, zinc-finger E-box-binding (ZEB) and basic helix-loop-helix transcription factors, the functions of which are finely regulated at the transcriptional, translational and post-translational levels. A characteristic example of EndMT constitute the transdifferentation of endocardial endothelial cells and their incorporation into the atrioventricular cushions. EndMT involves the modulation of a variety of signaling axes including TGF-β, Notch and Wnt pathways, as well as the acquisition of a new transcriptional program, in which EndMT effectors downregulate the expression of several endothelial markers, such as platelet EC adhesion molecule-1 (Pecam-1), vascular endothelial (VE)-cadherin, vascular endothelial growth factor receptor (VEGFR), and the angiopoietin receptor Tie-2, and upregulate the expression of mesenchymal proteins, including fibroblast specific protein-1 (FSP-1), α-smooth muscle actin (αSMA) and fibronectin (Sánchez-Duffhues et al., 2018).

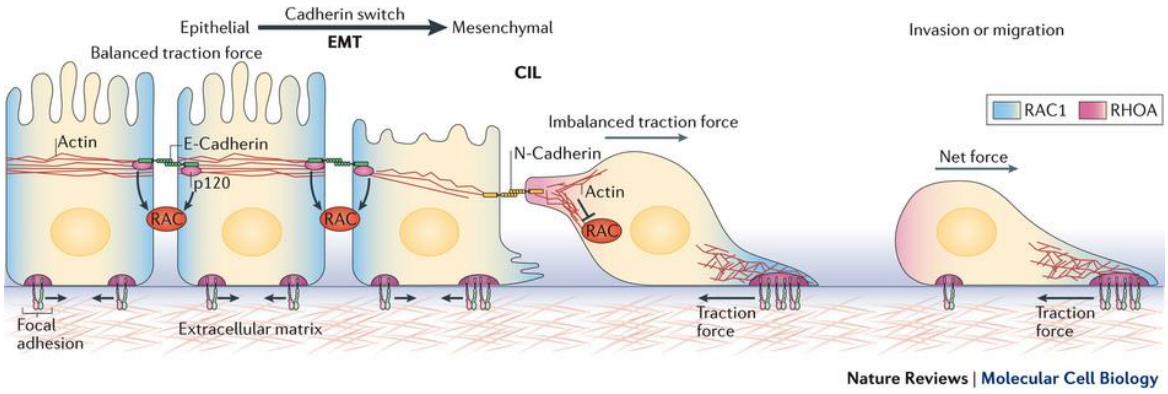


Figure 1.1.7.i

Loss of epithelial cell polarity during EMT.

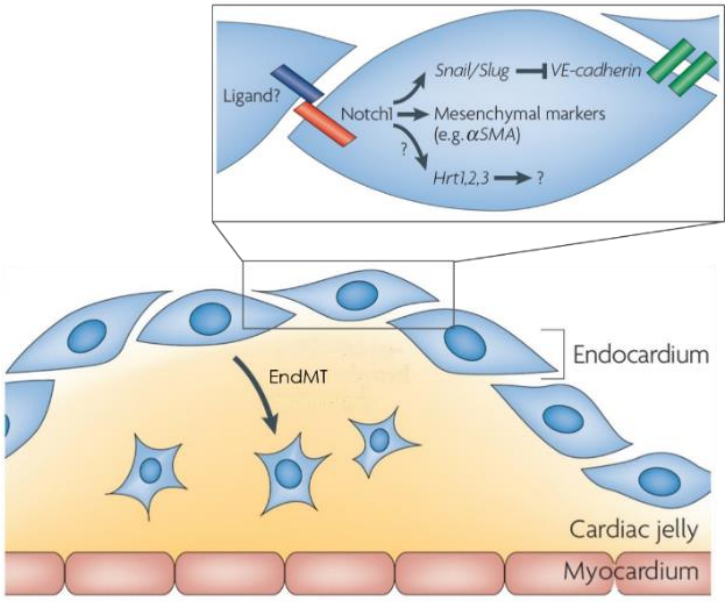


Figure 1.1.7.ii

Endothelial-to-Mesenchymal Transition.

From High and Epstein, 2008

1.1.8 Vesicular transport in cell polarity

Sorting of transmembrane proteins to the apical and basolateral surfaces necessitates two types of vesicular transport carriers emanating from the trans-Golgi network (TGN) and recycling endosomes. Trans-Golgi network participates in the sorting of newly synthesized proteins, whereas recycling endosomes are involved in returning internalized proteins to the cell surface. Polarized sorting depends on recognition of specific motifs on the cargo proteins. Basolateral signals are located in the cytosolic domains of the proteins and in some cases consist of amino acid arrays with specific motifs. In some other cases, the basolateral signal is a noncanonical sequence, such as the noncanonical sequence GDNS of the transferrin receptor (TfR). Mutations in these signals abrogate basolateral sorting, resulting in cell polarity perturbations. In Drosophila, in the pIIb daughter cell of sensor organ precursors (SOP), Numb inhibits the ability of the adaptor protein AP-1 to return Sanpodo to the basolateral surface, a function mediated by the YTNPAF sequence in the Sanpodo N-terminal tail, which binds to the Numb PTB domain. Redistribution of Sanpodo to the plasma membrane by mutation of genes encoding Numb or AP-1 subunits leads to Notch activation,

resulting in pIIb daughter cell, implying that some adaptors, such as Numb counteract the ability of other adaptors, such as AP-1 to promote transport. [(Bonifacino et al. 2014), (Figure 1.1.8.i)

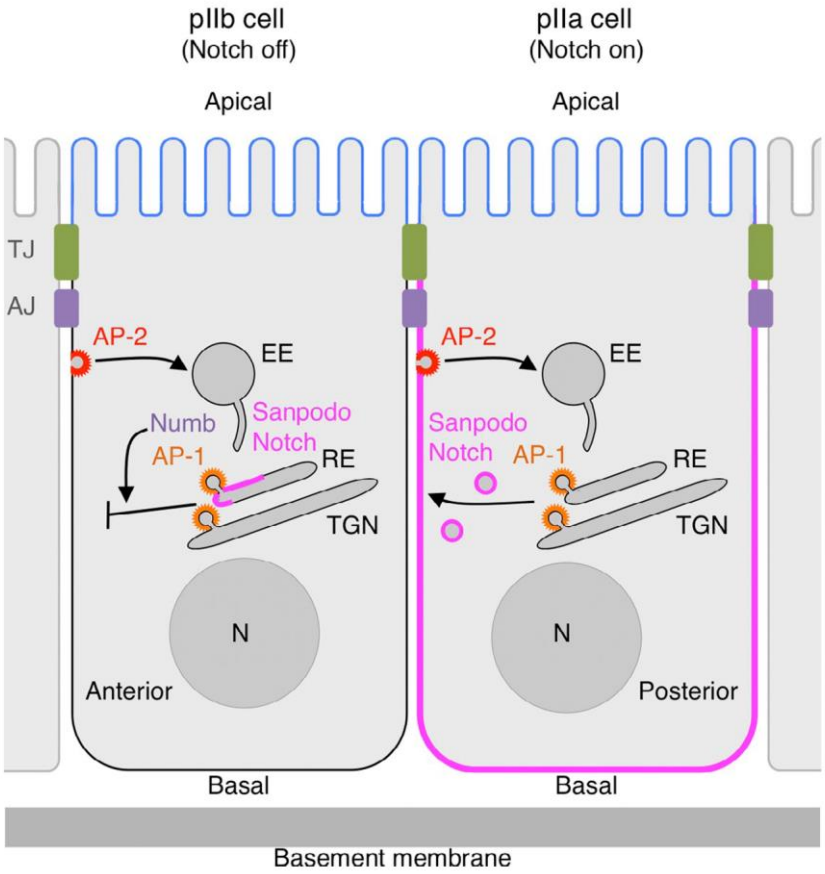


Figure 1.1.8.i

AP-2 complex mediates Notch and Sanpodo internalization, and AP-1–dependent recycling of Sanpodo and Notch in the pIIa cell is abrogated in the presence of Numb.

Polarity determinants play a key role in endosomal sorting and are characterized as master regulators of receptor trafficking. More specifically, in *Drosophila*, loss of the endocytic machinery resembles loss of the basolateral cell polarity proteins Lgl, Dlg, or Scrib. Scrib facilitates BMP-typeI receptor (Tkv) internalization to Rab5 endosomes, where Tkv is active. In erythroid progenitors, under conditions of high iron, TfR2-Scrib-associated trafficking vesicles are routed predominantly to the cell surface and minimally to lysosomes, therefore lysosomal catabolism of TfR2–Scribble complexes occurs at a low, basal rate. Maintenance of Scrib levels permits Scrib interaction with EpoR in receptor vesicles and Scrib-mediated presentation of EpoR to the cell surface.

Under conditions of low iron, TfR2-Scrib-associated vesicles are routed predominantly to lysosomes, accelerating catabolism of TfR2–Scribble complexes (Figure 1.1.8.ii). As a result, Scrib levels are too low, impairing the interaction of Scrib with EpoR and Scrib-mediated cell surface delivery of EpoR (Khalil et al., 2018). Scrib is required to maintain normal integrin α5 recycling and prevents its lysosomal degradation pathway mediated by Rab7a which controls the sorting from late endosomes into lysosomes (Michaelis et al., 2013).

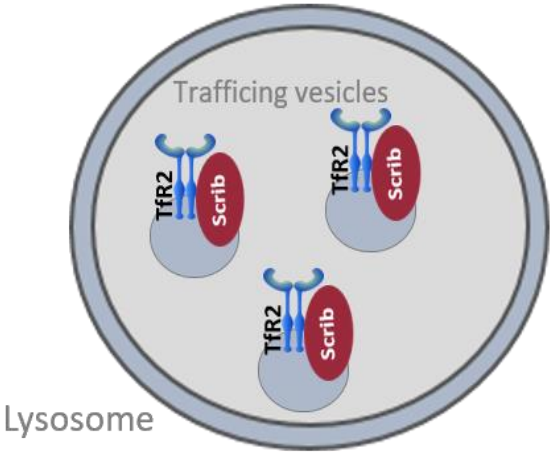


Figure 1.1.8.ii

TfR2-Scrib lysosomes, under low iron conditions.

In hippocampal neurons, Scrib1 is proven to stabilize GluN2A subunit-containing *N*-methyl-D-aspartate receptors NMDARs (ligand-gated ion channels) in basal conditions. Upon the activation of NMDARs, Scrib1 leads to the entry of GluN2A containing NMDARs into the recycling pathway, preventing lysosomal trafficking and degradation by increasing their recycling to the plasma membrane through a direct interaction with the AP2 adaptor proteins and the use of ARF6 regulatory proteins following NMDAR stimulation but prevents the trafficking of GluN2B-containing NMDAR. Downregulation of Scrib1 in hippocampal neurons leads to a decrease in synaptic NMDAR currents [(Piguel et al., 2016), (Figure 1.1.8.iii)]. SCRIB regulates steady-state cell surface levels of PRLR, as SCRIB knockdown results in PRLR translocation from the plasma membrane to the Golgi and to RAB-11 recycling endosomes (Baker et al., 2016).

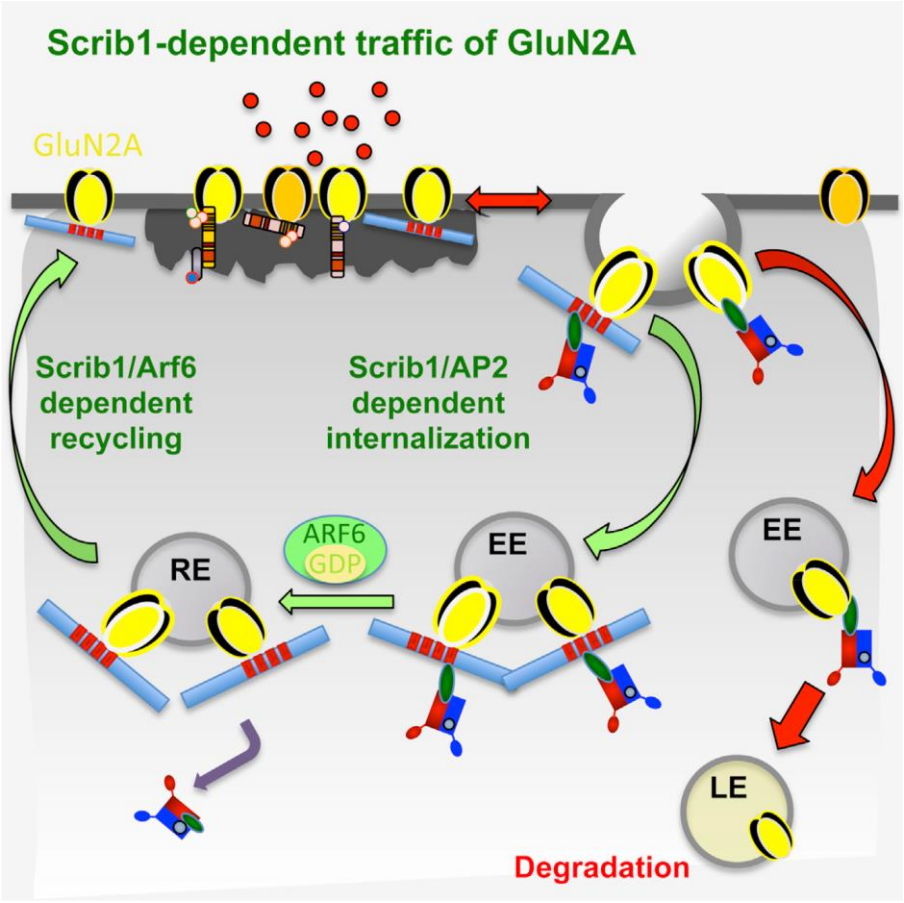


Figure 1.1.8.iii

Scrib1-dependent recycling of NMDARs.

In Drosophila, Numb localizes at endosomes and regulates the endosomal sorting of Notch, inhibiting its recycling in the context of asymmetric cell division in sensory organ precursor cells (SOPs) (Coutourier et al., 2013). Numb contains a phosphotyrosine-binding (PTB) domain, proline-rich regions (PRRs) and tripeptide sequence motifs such as Asn-Pro-Phe (NPF) and Asp-Pro-Phe (DPF). It has been shown to take part in clathrin-dependent endocytosis at the plasma membrane, interacting with Eps15 through its NPF motif, as well as with a subunit of AP-2 adaptor complex, the α-adaptin through its DPF motif. The PTB domain of Numb bind several transmembrane proteins, such as Notch, APP and β-integrin and is thought to function as an adaptor protein that selects specific cargo and links to endocytic machinery (Figure 1.1.8.iv).

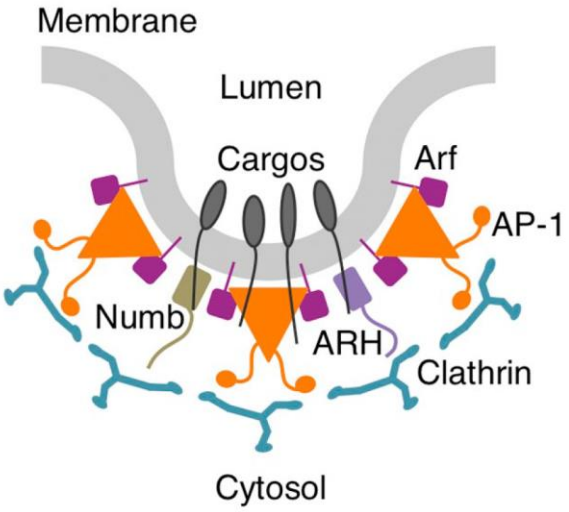


Figure 1.1.8.iv

The fate determinant Numb as an adaptor in clathrin-mediated trafficking.

1.1.9 Different modes of cell polarity during mammary gland development

During the lifetime of the female, the mammary gland undergoes many changes in tissue architecture (Figure 1.1.9.i), and function, resulting from microenvironmental cues, hormonal changes induced by the estrous/menstrual cycle, as well as changes that occur during pregnancy, lactation and involution (Figure 1.1.9.iii). During these different stages, the cells of the mammary gland undergo changes in cell polarity, proliferate, differentiate, migrate and apoptose in response to stimuli. Asymmetric cell divisions resulting in cell fate specification, epithelial cell polarity, planar cell polarity and migratory polarity are the major types of cell polarization acquire the cells of mammary gland during development. During puberty, the mammary ductal tree elongates under the influence of estrogen and invades into the mammary fat pad. Terminal end buds (TEBs) are characteristic mammary structures of the pubertal stage. In adulthood, the mammary ductal system is expanding through progesterone-induced side branching. Side branching is enhanced during early pregnancy. In pregnancy alveoli develop and they are filled with milk during lactation. It is proven that the polarity protein SCRIB is a positive regulator of cell proliferation during pregnancy-associated mammary alveologenesis (Baker et al., 2016) (Figure 1.1.9.ii). Multiple epithelial cell types can be found in the mammary gland. The mammary bilayer found throughout the adult gland of virgin mice is composed of apically

oriented luminal epithelial cells that line the ducts and of basally oriented myoepithelial cells in contact with the basement membrane (Inmnan et al., 2015).

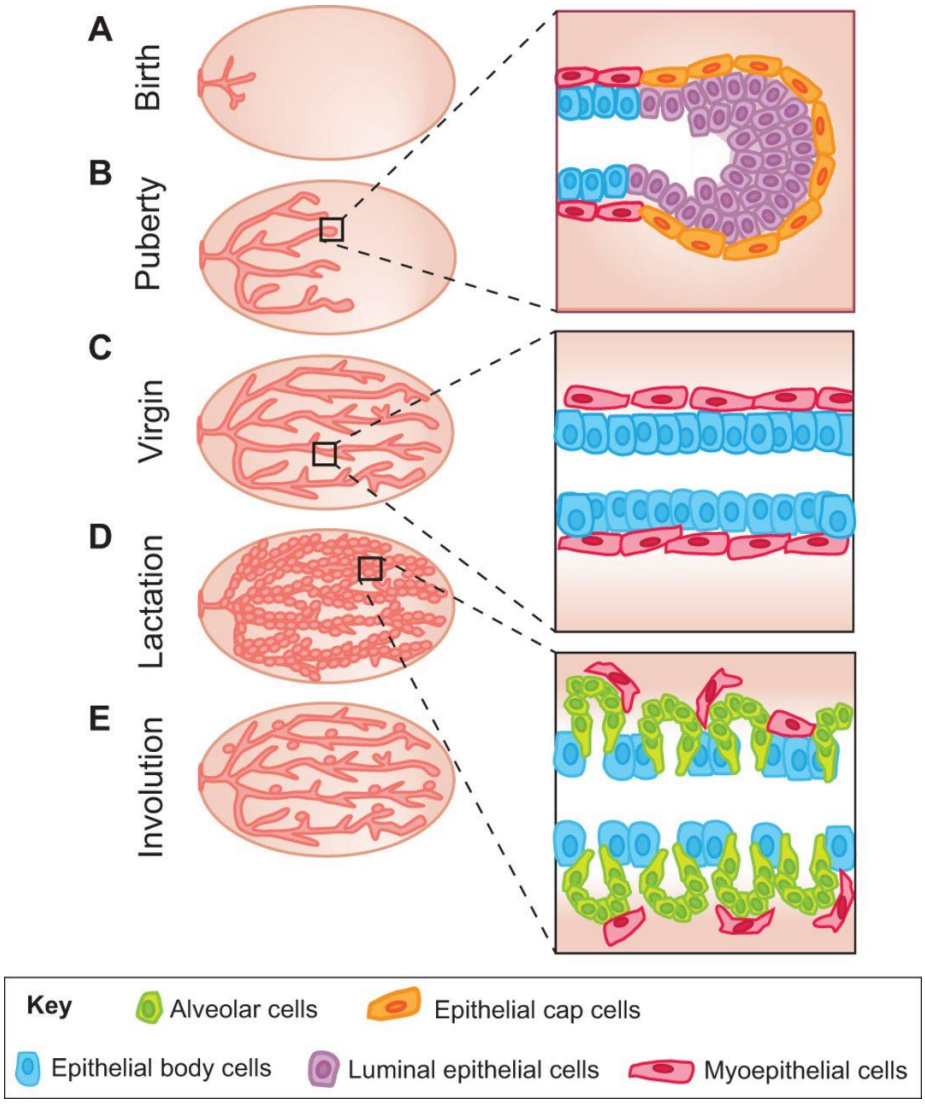


Figure 1.1.9.i

The multistage process of mammary gland development after birth. From Inman et al., 2015.

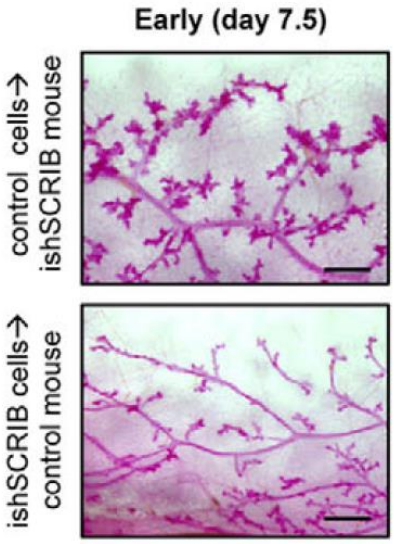


Figure 1.1.9.ii

SCRIB is required for expansion of epithelial cells during pregnancy-induced alveologenesis. From Baker et al., 2016.

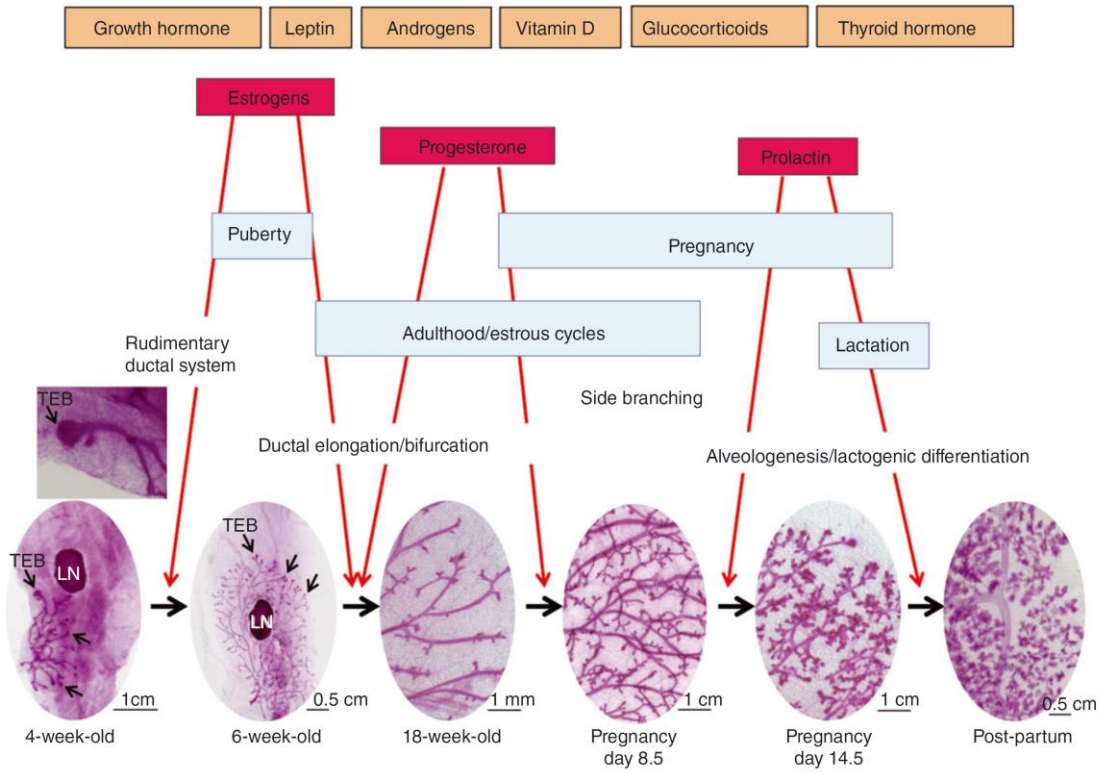


Figure 1.1.9.iii

Hormonal control of mouse mammary gland development.

From Brisken et al., 2015.

1.2 Polarity proteins in cancer

Polarity proteins regulate temporally and spatially cell signaling. The regulation of their cellular localization play pivotal role in their function. They all contain protein-protein interaction domains that allows the interaction with and binding to signaling proteins that regulate cell growth, differentiation, apoptosis, cell adhesion, membrane trafficking and epithelial-mesenchymal transition (EMT). Apical–basal polarity is established and maintained by evolutionarily conserved modules of multi-domain proteins, such as Par, Crumbs and Scrib module.

The scaffolding protein Par3, the adaptor protein Par6, the atypical protein kinase C (aPKC), and the small GTPase Cdc42 constitute members of the Par module. Par3 binds to phospholipids of the plasma membrane and associates with the tight junction protein JAM-A. Furthermore, Par3 recruits Par6 and aPKC to the plasma membrane where the Cdc42-mediated conformation change of Par6 leads to aPKC activation. Parb1 resides to the basolateral domain of epithelial cells where it excludes the localization of Par3, Par6 and aPKC. More specifically, Par1b phosphorylates Par3, this phosphorylation event creates a binding site for 14-3-3 proteins, and results in dissociation of Par3 from the cell cortex. Whereas, aPKC phosphorylates Par1b to exclude it from the apical domain. Liver kinase b1 Lkb1 phosphorylates and activates AMPK, which in turn is implicated in modulating cell polarity by regulating tight junction formation. (Figure 1.2i)

In epithelial cells, the Crumbs complex is comprised of the transmembrane protein Crumbs 3 (Crb), which binds the multi-domain proteins Pals1 (MPP5), Pals1-associated tight junction protein (INADL), and angiomotin (Amot), that are required for Tight Junction formation as well as apical membrane specification. (Figure 1.2.ii)

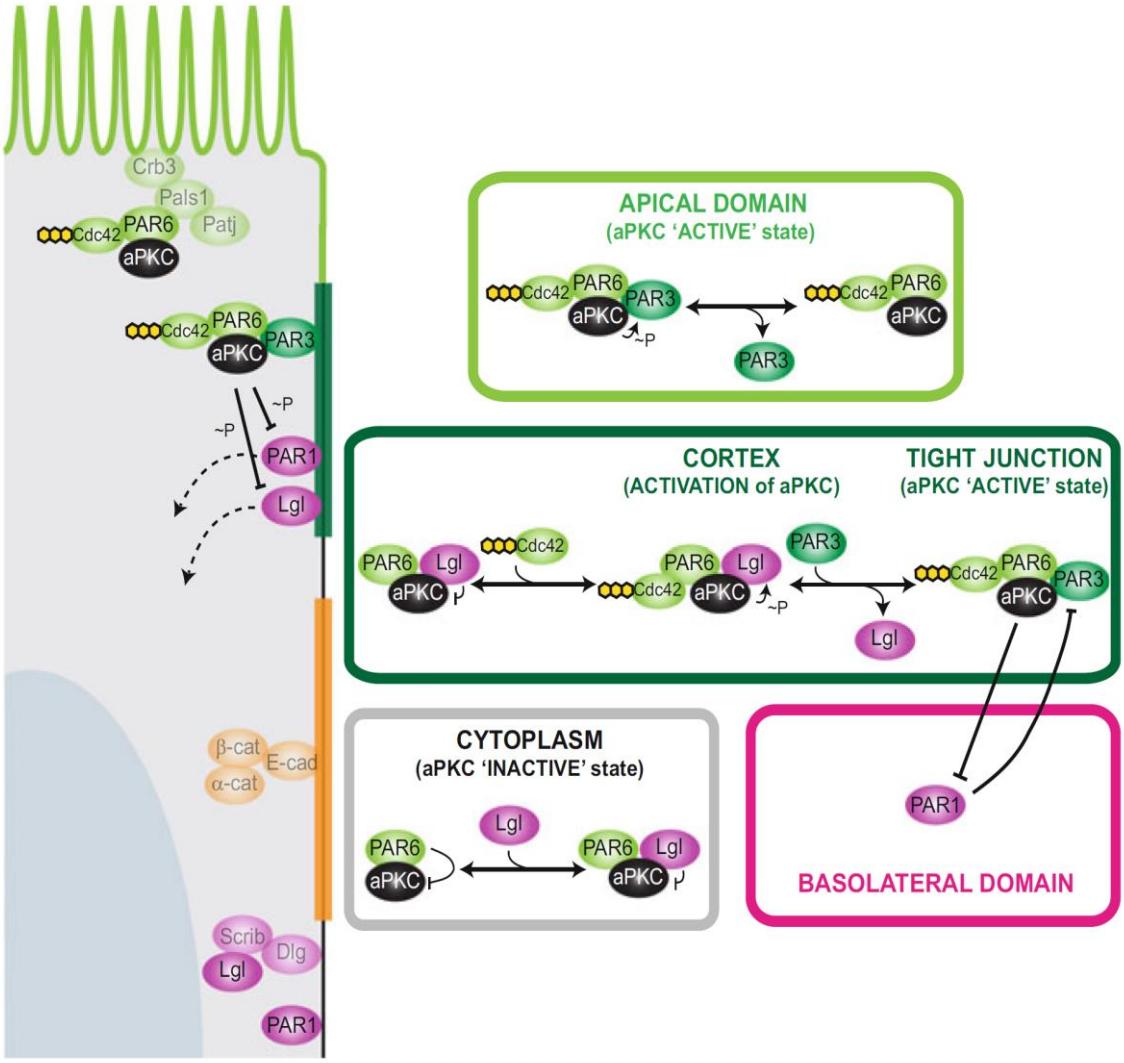


Figure 1.2.i

The PAR module in epithelial cell polarity.

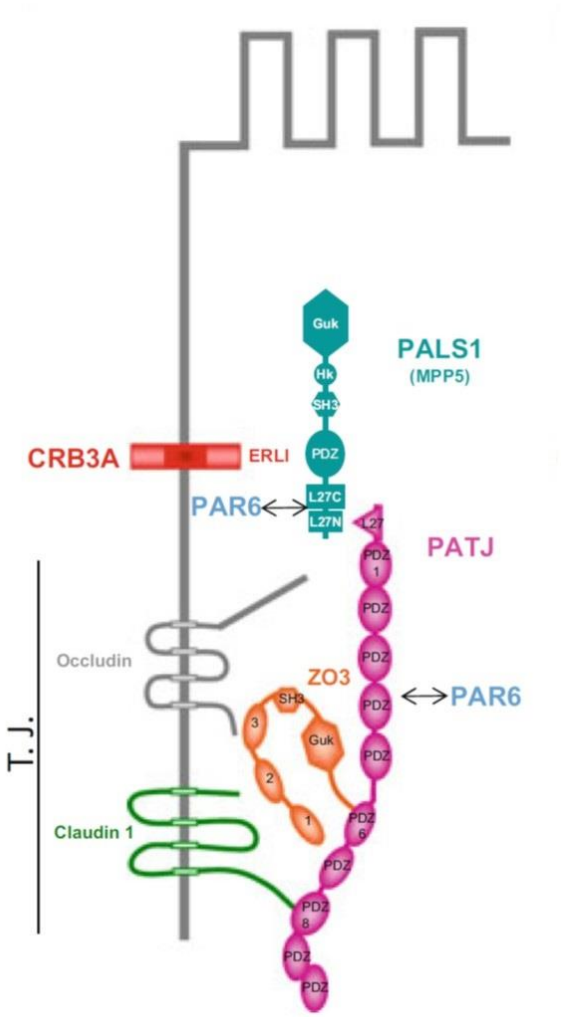


Figure 1.2.ii

The Crumbs complex in epithelial cell polarity.

The Scribble module resides at the basolateral membrane and consists of the scaffolding proteins Scribble (Scrib), Discs-large (Dlg), and the adaptor protein lethal giant larvae Lgl2. The basolateral membrane localization of Scrib module is dependent on cell–cell adhesion mediated by E-cadherin, whereas at the same time, the Scrib module is necessary to maintain E-cadherin-mediated adhesions.

The three polarity complexes act in a complementary manner in the establishment and maintenance of cell polarity. More specifically, Lgl2 associates with Par6 and aPKC, which is important for trafficking of Par6/aPKC proteins. Par3 competes with Lgl2 for binding, whereas activated aPKC phosphorylates and inhibits Lgl2. Moreover,

Par6 can interact with Pals1 and Crb, and Pals1 is required for normal localization of the Par complex. Crb competes with Par3 for binding Par6, which localizes Par6/aPKC to the apical membrane in polarized cells.

1.2.1 Scrib module

The Scrib module comprises of three distinct members, Scribble (Scrib), Lethal giant larvae (Lgl), as well as disk large (Dlg) and each member has been shown to associate with another. In mammals, Scrib plays a key role in establishment and maintenance of apico-basal and planar cell polarity. The members of the Scrib polarity module are large proteins that bear protein–protein interaction domains. Scrib belongs to LAP family proteins and contains 16 Leucine-Rich Repeats LRR, a protein motif that consists of repeated 20–30 amino acids, four PDZ (PSD-95/Dlg/ZO-1 homology) domains, a protein motif that consists of 80–90 amino acids and mediate protein–protein interactions generally through binding to peptide motifs at the C-terminus of proteins, as well as three SADH spectrin-binding domains (Figure 1.2.1.ii). Discs large (Dlg) is a member of the MAGUK family of proteins and contains 3 PDZ domains, an Src homology 3 (SH3) domain, as well as guanylate kinase-like (GUK) domains. The catalytically inactive GUK domain of MAGUK proteins is thought to play a role in protein interactions, while the SH3 domain is a protein–protein interaction domain, specialized in recognition of proline-rich motifs and can promote the assembly of large multiprotein complexes. Lgl contains four WD40 repeats that are structural motifs composed of 40–60 amino acids that usually end with a tryptophan–aspartic acid (WD) dipeptide and assemble in four-stranded antiparallel sheet conformation.

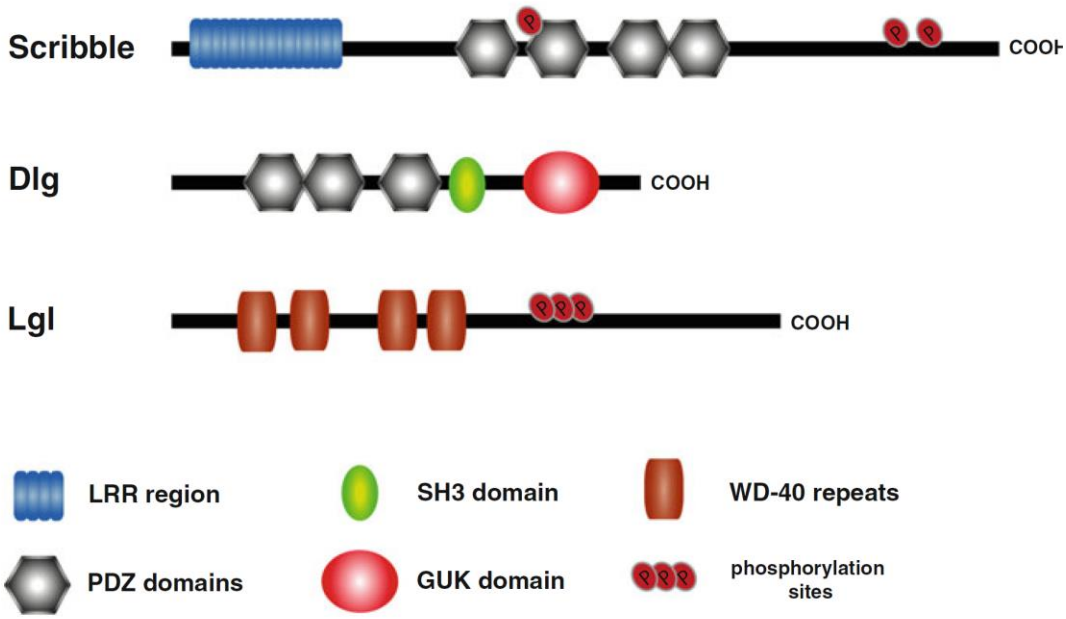


Figure 1.2.1.i

Structural representation of the members of Scrib module.

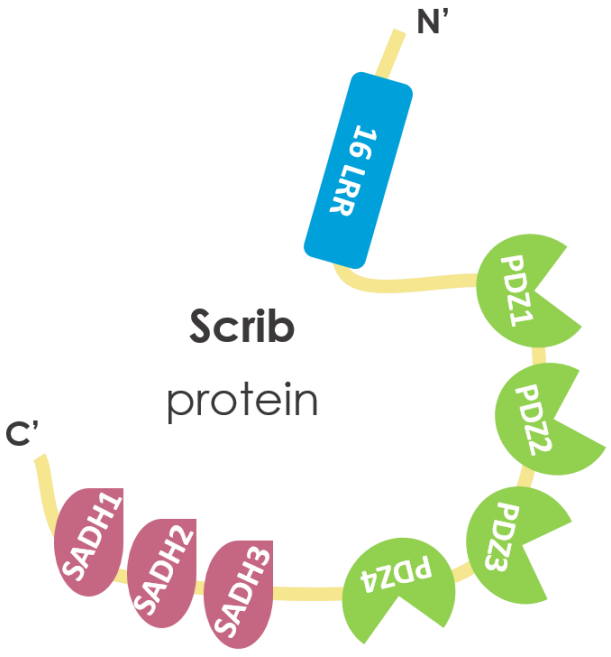


Figure 1.2.1.ii

The LRR, PDZ and SADH domains of Scrib.

1.2.1.1 Biological function of Scrib protein

The polarity protein Scrib play a key role in several crucial cell functions. It is well established that Scrib function in cell-cell adhesions. For instance, it has been shown that Scrib is settled to adherens and tight junctions of lung epithelia and knockdown of Scrib in lung explants and organotypic cultures leads to reduced cell-cell association of lung epithelial cells. Furthermore, in MDCK cells, Scribble knockdown results in delayed tight junction assembly and impaired recruitment of E-cadherin to the membrane (Qin et al., 2005). Moreover, Scrib has been characterized to play a key role in morphogenesis and differentiation. In mice mammary gland, SCRIB is expressed in the E-cadherin-positive luminal epithelial cells, but not in in the cytokeratin 14 or KRT5-positive basal epithelial cells of ducts and terminal end buds, and it is required for pregnancy-induced alveolar proliferation (Baker et al. 2016). Additionally, Scrib is necessary for normal lung morphogenesis and disruption of Scrib perturbs epithelial tube formation, leading to perturbation in cytoskeletal organization via downregulation of PCP-RhoGTP signaling pathway (Yates et al., 2013).

Scrib has also been characterized as a cell fate determinant during myogenesis. Stem cell myogenesis is a multi-stage process characterized by the expression of certain combinations of myogenic factors in the stages of activation, asymmetric cell division, proliferation and in the later stage of commitment. Quiescent muscle satellite skeletal stem cells do not express Scrib whereas upon their activation the expression of Scrib increases during myogenic progression. Scrib protein tends to be highly segregated into daughter cells that undergo myogenic differentiation and it is low expressed in proliferating or self-renewing cells. Furthermore, Scrib permits activated satellite cells to expand the progenitor population for appropriate muscle regeneration. (Ono et al., 2015)

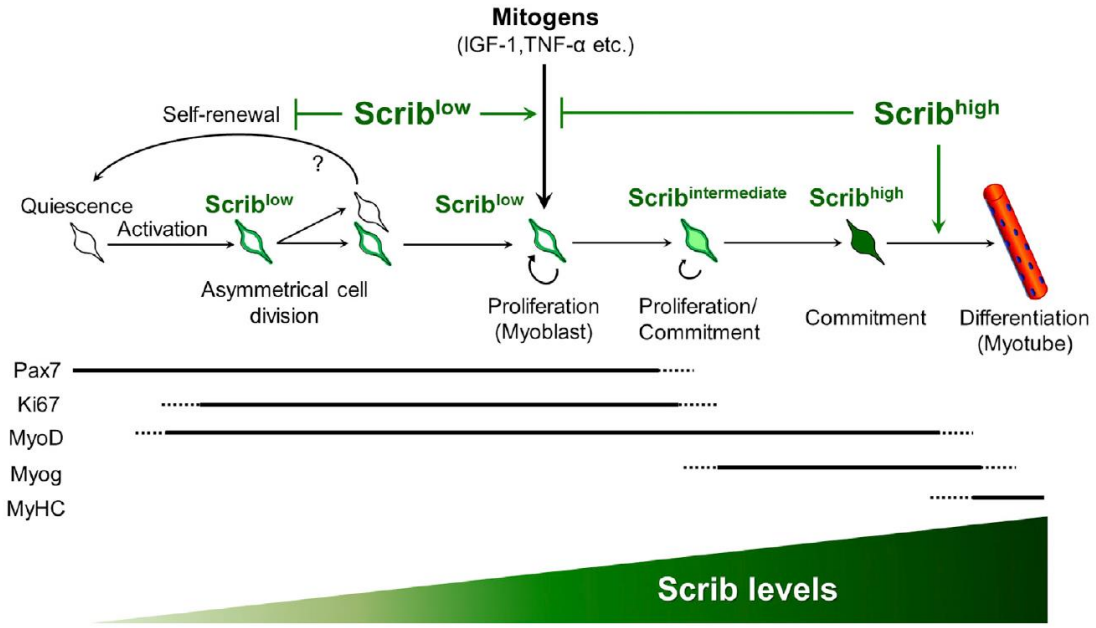


Figure 1.2.1.1.i
Schematic of expression patterns of Scrib protein during myogenic progression in satellite cells. From Ono et al., 2015

In normal mammary development, Scrib moderates basal progenitor clonogenic potential and controls spindle orientation to maintain a balanced epithelial hierarchy. Scrib also play a key role in remodelling and establishment of apical-basal polarity during late stage duct morphogenesis. Scribble loss leads to hyperactivation of the Ras/MAPK pathway and the expansion of ectopic luminal epithelial cells to promote tumour development (Figure 1.2.1.1.ii). (Godde et al., 2014)

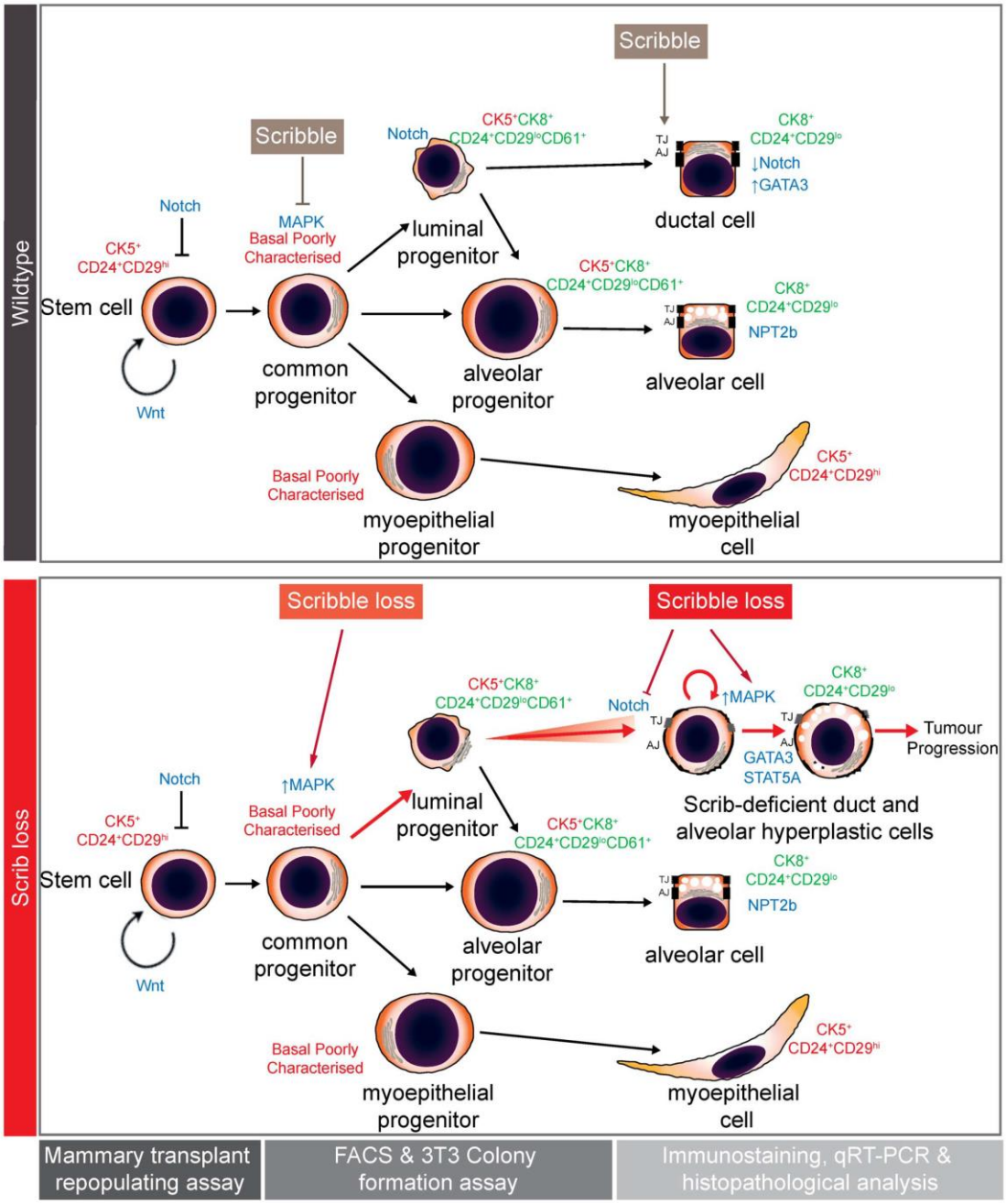


Figure 1.2.1.1.ii
Scrib in mammary development and homeostasis. From Godde et al., 2014.

Yet another function of Scrib is to regulate Epithelial-to-Mesenchymal Transition (EMT). Scrib downregulates ZEB by inhibiting the ERK1/2 signalling axis. ERK2 signaling is necessary for Ras-induced Epithelial-to-Mesenchymal Transition (EMT), regulating the expression of the zinc transcription factor ZEB through the accumulation and activation of Fos related antigen-1 (Fra1) in human breast epithelia (Shin et al., 2010).

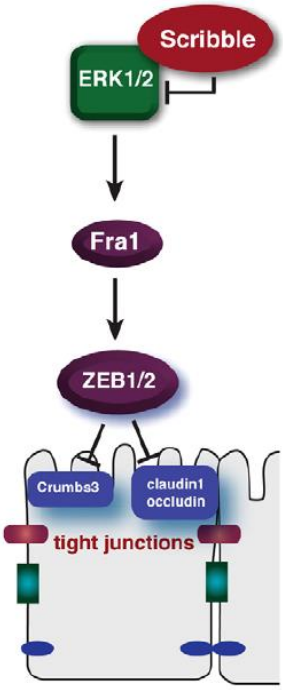


Figure 1.2.1.1.iii
Scrib functions in MAPK-ERK signaling.
From Elsum et al., 2013.

Furthermore, Scrib1 has been shown to play a role in neuronal function, being localized postsynaptically at NMDA excitatory synapses in the stratum radiatum in CA1 of the hippocampus as well as in intracellular vesicles or organelles in the dendritic spine, regulating NMDA recycling (Piguel et al., 2016).

It is proven that Scrib plays a crucial role in apoptosis. More specifically, it has been shown that in MDCK epithelial cells cultured at confluent density, βPIX knockdown results in apoptotic cell death and the concomitant knockdown of Scrib rescues cell from apoptosis. Expression of a chimeric protein consisting of the LRR region of Scrib fused with a constitutively active PAK2 is capable to protect anoikis in βPIX-depleted cells, whereas the expression of the LRR domain alone enhances apoptotic cell death in βPIX-knockdown cells, suggesting that this domain is sufficient to confer sensitization to apoptosis. Overexpression of the LRR domain of Scrib in MDCK cells with normal levels of βPIX had no effect on cell viability. Targeting of PAK2 to adherens

junctions, via a βPIX-dependent association with Scrib, is required to counteract apoptosis sensitization elicited by Scrib and E-cadherin (Frank et al. 2012).

1.2.1.2 Subcellular localization of Scrib

The subcellular localization of Scrib is crucial for its function. It is well established that the LRR domain of Scrib is necessary to attach Scrib to the plasma membrane (Su et al. 2012). The LRR mislocalization Scrib mutant (P305L) fails to suppress MAPK signaling and invasion (Elsum et al. 2013). Mutations affecting the PDZ region (P1043L) have been reported to affect subcellular localization of Scrib as well (Robinson et al. 2011, Lei et al. 2013). Additionally, it has been proven that CD74 amplification in epithelial cancer leads to Scrib phosphorylation and mislocalization to the cytosol (Metodieva et al. 2013). Scrib is subjected to posttranslational modifications, regulating its temporal and spatial localization. More specifically, palmitoylated Scrib has been identified in MCF10A human epithelial cells as well as in highly aggressive T cell hybridoma cells. Palmitoylation of Cys-4 and Cys-10 was found to be necessary for its membrane recruitment, supporting the key role of palmitoyltransferases in directing polarized localization of cytosolic proteins (Chen et al., 2016, He et al., 2014). Also, zDHHC7 has been reported to participate in Scrib plasma membrane localization (Chen et al., 2016).

The subcellular localization of Scrib to organelles has been described. More specifically, Scrib localizes to early endosomes in *Drosophila* Wing posterion crossvein, where it forms a complex with the BMP receptor (Tkv) and Rab5 GTPase (Gui et al., 2016). On bacterial infection, Scrib localizes to phagosomes in a LRR-dependent manner, where it interacts directly with NOX complex and promotes ROS production within phagosomes to kill bacteria (Zheng et al., 2016).

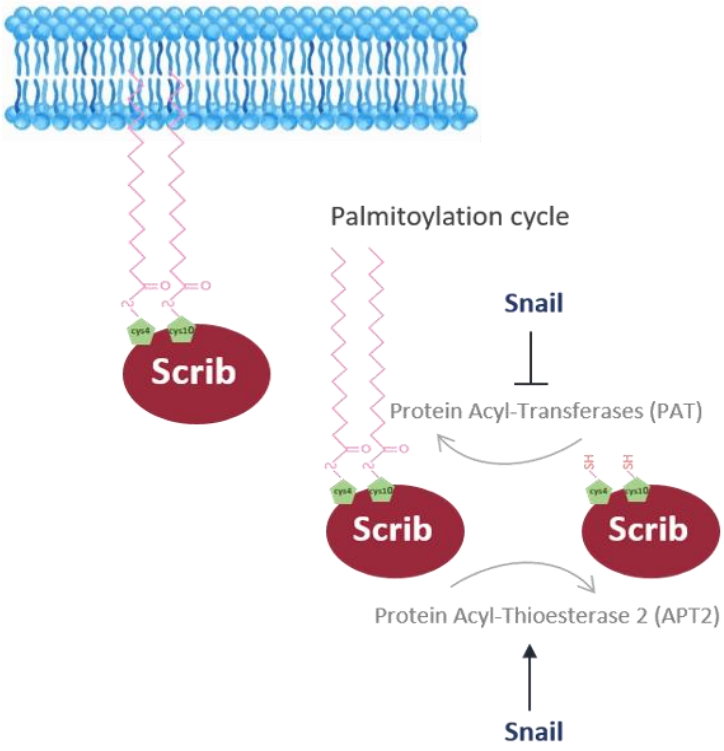


Figure 1.2.1.2.i
The palmitoylation cycle of Scrib.

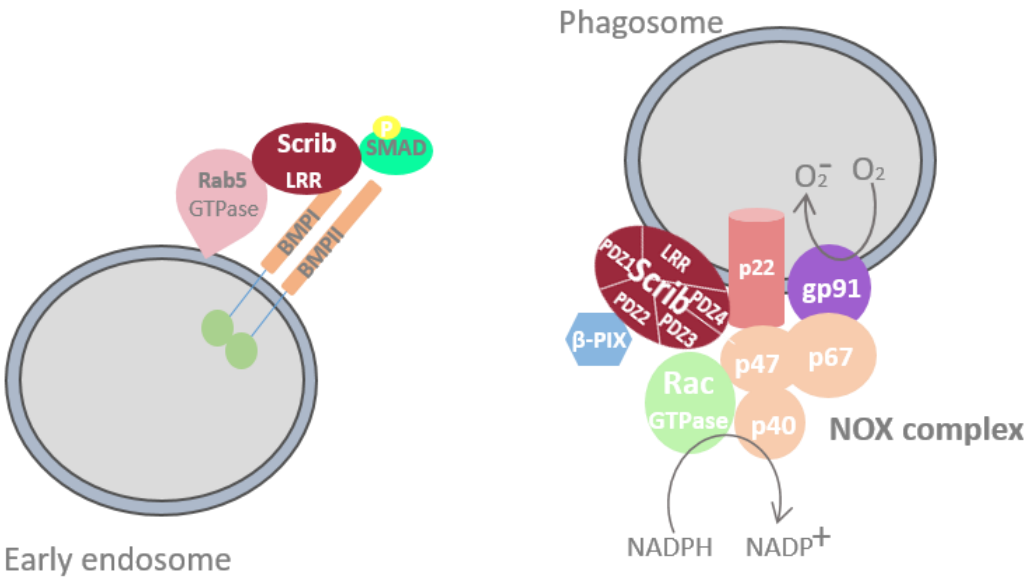


Figure 1.2.1.2.ii
Scrib localization to early endosomes and phagosomes in *Drosophila* and macrophages respectively.

1.2.1.3 Scrib interactors

Scrib binding to interacting proteins is mediated by affinity of protein binding either to a particular domain or to multiple domains as well as phosphorylation-dependent binding and palmitoylation-dependent binding. Several interactions of Scrib with adhesion, cell signaling and scaffold molecules have been reported. For instance, it is proven that in endogenous lung tissue, β-catenin and Scrib proteins physically interact. Also, TfR2 is proven to bind Scribble and to mediate its lysosomal catabolism in the iron deprivation response. Additionally, Scribble interacts with EpoR and promotes its cell surface delivery (Khalil et al., 2018). Integrin α5 has been identified as a Scrib interacting protein in endothelial cells that supports integrin α5 recycling and protein stability by blocking its interaction with Rab7a. Leucine rich repeat domain mutants does not restore integrin α5 abundance, implying the involvement of the LRR domain of Scrib to integrin a5 regulation (Michaelis et al., 2013). In hippocampal neurons, Scrib1 interacts with GluN2 subunits of NMDA receptors via its PDZ domains 2 and 3, as well as with the AP2 adaptor complex which play a crucial role to cargo internalization in clathrin-mediated endocytosis. It is reported that the Scrib1-AP2 interaction consists a key point to the endocytic sorting of NMDA receptors (Piguel et al., 2014). Furtermore, Scribble exists in a complex with the Arf-GAP GIT1, a known positive modulator of MEK-ERK signalling (Audebert et al., 2004, Yin et al., 2004) and thus could act as a negative regulator of GIT1 activity. Scribble binds the planar cell polarity proteins Vangl1 and Vangl2, which are required for proper Scribble localization.

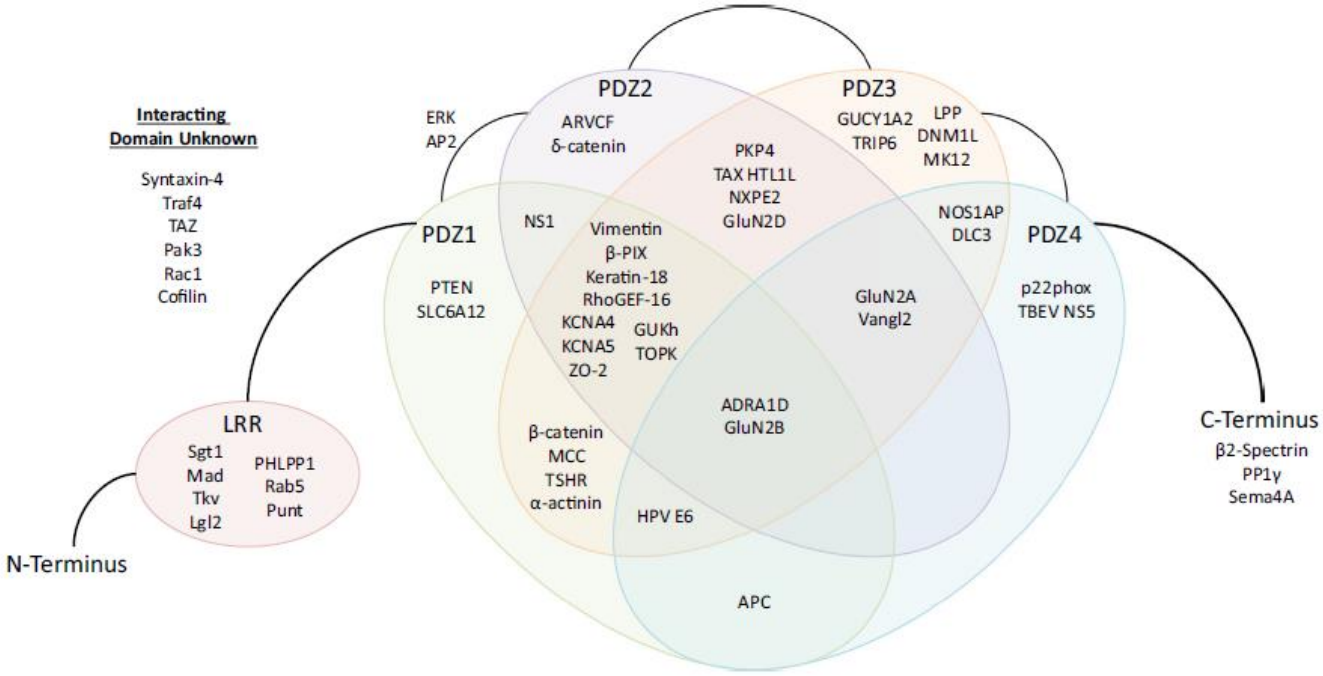


Figure 1.2.1.3.i
Interactors of Scrib protein. From Stephens et al. 2018.

1.2.1.4 Interplay of Scrib module with signalling pathways

The polarity proteins of the Scrib module physically interact with signalling components of several signaling pathways, regulating their activation state (Figure 1.2.1.4.ii). Studies in mammalian systems have shown that Scrib negatively regulates the Ras-MAPK signalling pathway in vitro and in vivo (Pearson et al., 2011, Elsum et al., 2013). The Ras–MAPK signaling pathway acts downstream of RTKs to regulate cell growth proliferation during development and homeostasis, and its deregulation occur frequently in cancer. The localization of the EGFR to basal-lateral domain of epithelial cells, restricts pathway activation to the baso-lateral domain. Scrib interacts indirectly with EGFR, by binding to the Arf-GAP, GIT1, and β-PIX (Pak-interactive exchange factor) that act as a MEK-ERK scaffold (Audebert et al., 2004). β-PIX together with the small GTPase, Cdc42, regulates EGFR plasma membrane levels by blocking Cbl E3-ubiquitin ligase-mediated ubiquitination of the EGFR. Scrib through its binding to β-PIX, might function in EGFR downregulation.

Scrib also interacts with downstream components of the Ras pathway, such as ERK and P90-RSK2 (ribosomal S6 kinase 2) (Zenniou-Meyer et al., 2008). P90-RSK2 is a serine/threonine-protein kinase that acts downstream of ERK (MAPK1/ERK2 and MAPK3/ERK1) signaling cascade. P90-RSK2 regulates translation through RPS6 and EIF4B phosphorylation, and mediates cellular proliferation, survival, and differentiation by regulating mTOR signaling and repressing pro-apoptotic function of BAD and DAPK1. Additionally, P90-RSK2 phosphorylates GSK3β at Ser-9 inhibiting its activity. Human Scrib inhibits Ras signaling by direct binding to ERK at the kinase interaction motif (KIM) docking sites and preventing ERK phosphorylation and by anchoring ERK to membrane sites (Nagasaka et al., 2010).

Scrib and Dlg also interact with phosphatases that impact on EGFR-Ras signaling. Dlg2 and Dlg3 interacts with the catalytic subunit of the PP1 phosphatase, whereas Scrib interacts with protein phosphatase 1γ (PP1γ), regulating ERK activity (Nagasaka et al., 2013). Scrib has also been shown to form a complex with SHOC2/SUR-8 (PP1 phosphatase regulator) and MRAS (RRas subgroup of Ras proteins). Scrib blocks SHOC2-mediated dephosphorylation of RAF (a protein kinase downstream of Ras) at the conserved inhibitory site (S259), inhibiting RAF activation (Young et al., 2013).

In human MCF10A cells, Scrib knockdown leads to JNK activation, which contributes to the invasive phenotype with oncogenic Ras, H-RasV12, however the mechanism by which JNK is activated in these Scrib-deficient cells is unknown. Conversely, Scrib has a positive role in activating JNK in mouse models of c-Myc driven breast cancer. Here, Scrib by its ability to promote assembly of the βPIX/GIT1 complex and activate Rac1, which in turn induces the JNK/c-Jun pathway leading to the expression of pro-apoptotic Bim, promotes c-Myc induced apoptosis (Zhang et al., 2008).

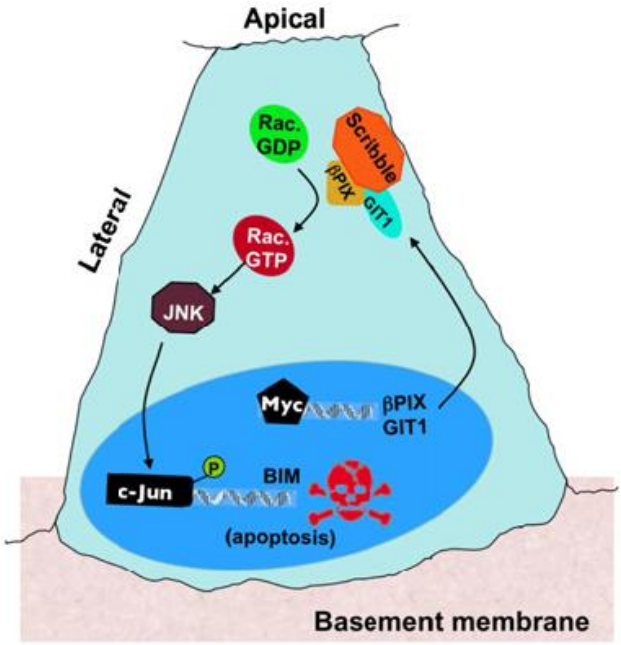


Figure 1.2.1.4.i

Scrib regulates c-Myc-induced apoptosis in polarized epithelial cells.

From Zhang et al., 2008.

Several studies support the involvement of the polarity protein SCRIB in Akt signalling axis. It has been shown that Scrib targets the protein phosphatase PHLPP to the membrane and negatively regulates AKT activity (Li et al., 2011). A link between SCRIB and mTOR signaling has been recently reported (Feigen et al., 2014), where the authors show that a mislocalized form of SCRIB when overexpressed, functions as a neomorph by interacting with PTEN, thus activating an Akt/mTORC1/S6K signaling cascade.

It has been proven that members of Scrib polarity module interact with several components of Wnt signalling. Wnt signaling is transduced either via the canonical pathway involving the transcriptional activity of β-catenin, or via non-canonical pathways, such as in PCP, which signal through the adaptor protein, Disheveled, and the Rho1-GTPase and JNK pathways. The canonical Wnt signaling pathway regulates the stability of β-catenin through inactivation of the APC–axin complex, which induces

ubiquitin-mediated degradation of β-catenin. Scrib interferes with Wnt signalling, interacting physically with the PCP gene, Vangl2, as well as with the PCP regulator LPP.

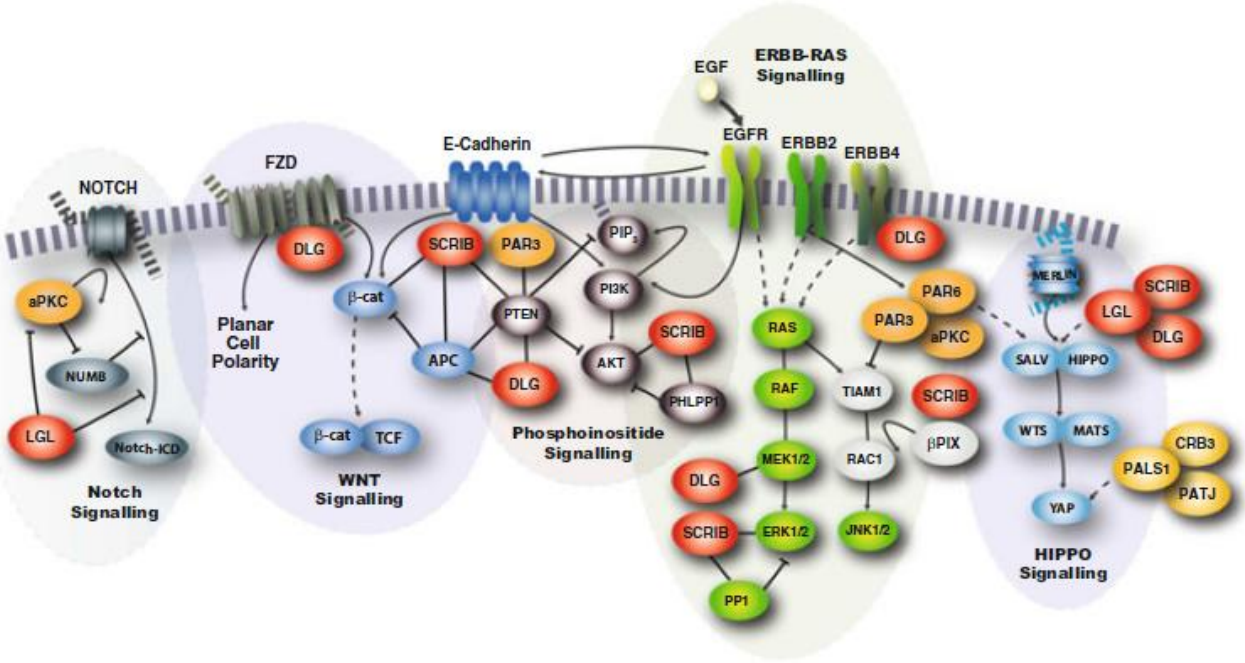


Figure 1.2.1.4.i

Crosstalk of Scrib module with signalling molecules.

1.2.1.5 Scrib expression in human tissues

The localization of Scrib in mammalian cells is tightly controlled and the abrogation of its proper localization results in cell apolarity and perturbations of tissue architecture. Tissue expression and localization of Scrib has been assessed in physiologic or pathologic histological samples. In normal colon specimens, in the lower part of the colon crypt, Scrib is expressed at low levels, displaying a basolateral distribution. As the cells move up from the bottom of the crypt toward the upper part, Scrib expression increases gradually, and an alteration of its sub-cellular localization is observed. In the luminal surface of the crypt, Scrib protein is abundant and diffuse, localized predominantly at regions of cell–cell contact and within the cytoplasm (Gardiol et al., 2006) (Figure 1.2.1.5.i).

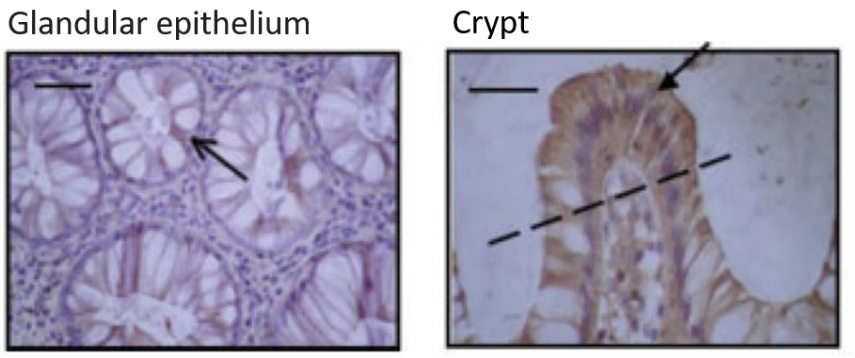


Figure 1.2.1.5.i

Immunohistochemical analysis of the expression and localization of hScrib in normal colon mucosa. From Gardiol et al., 2006

In human and mice hematopoietic tissue, SCRIB undergoes up-regulation at the first committed erythroid stage, corresponding to burst-forming unit-erythroid BFU-E, followed by down-regulation in subsequent stages (Figure 1.2.1.5.ii).

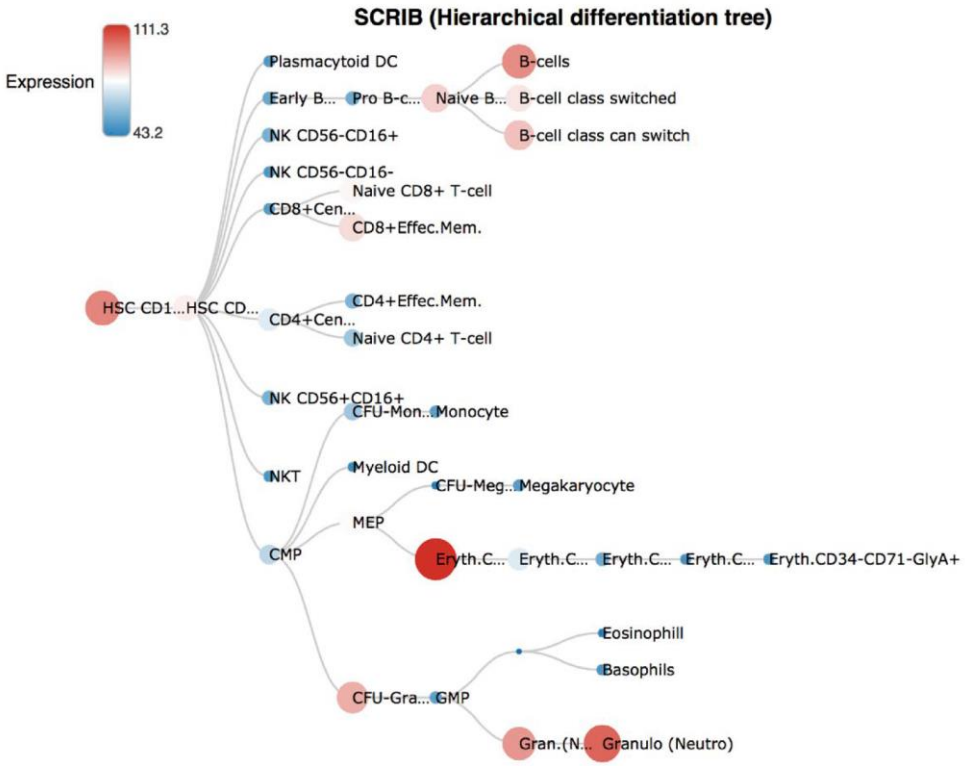


Figure 1.2.1.5.ii

Heat map of SCRIB expression levels in hematopoietic hierarchy from BloodSpot server using normal human hematopoietic DMAP dataset.

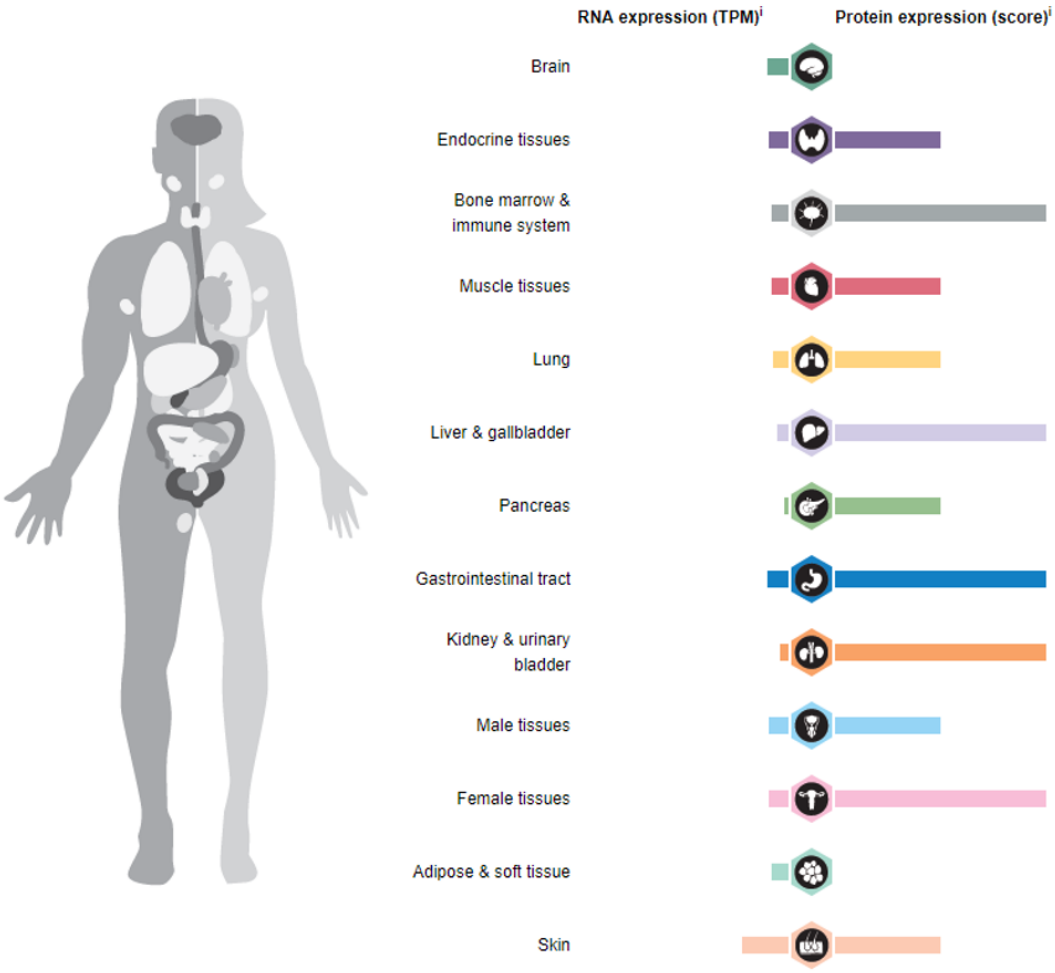


Figure 1.2.1.5.iii

Protein and RNA levels of Scrib protein in human tissues.

From https://www.proteinatlas.org/ENSG00000180900-SCRIB/tissue.

1.3 'Cell apolarity' or cell polarity of pathological conditions

Lack of cell polarization leads to tissue disorganization and result in disease, such as age-associated diseases and cancer (Soares et al. 2013, Martin-Bemonte and Perez-Moreno 2012). Several physiological processes based on cellular polarity are altered during aging. Age-associated changes have been found in polarity-determined biological functions, such as angiogenesis and epithelial barrier function. Decreases in the number of structural components of the polarized epithelial barrier have been observed in aging organisms, as well as alterations of the activation status of the signaling pathways RAF/MEK/ERK, GSK-3 and AMPK that regulate those structural

components or the assembly of the tight junctions may also be related with the age-related changes. Furthermore, mammalian cells during aging accumulate misfolded and oxidatively damaged proteins, increasing the pool of aged cells (Figure 1.3.i). (Soares et al. 2013)

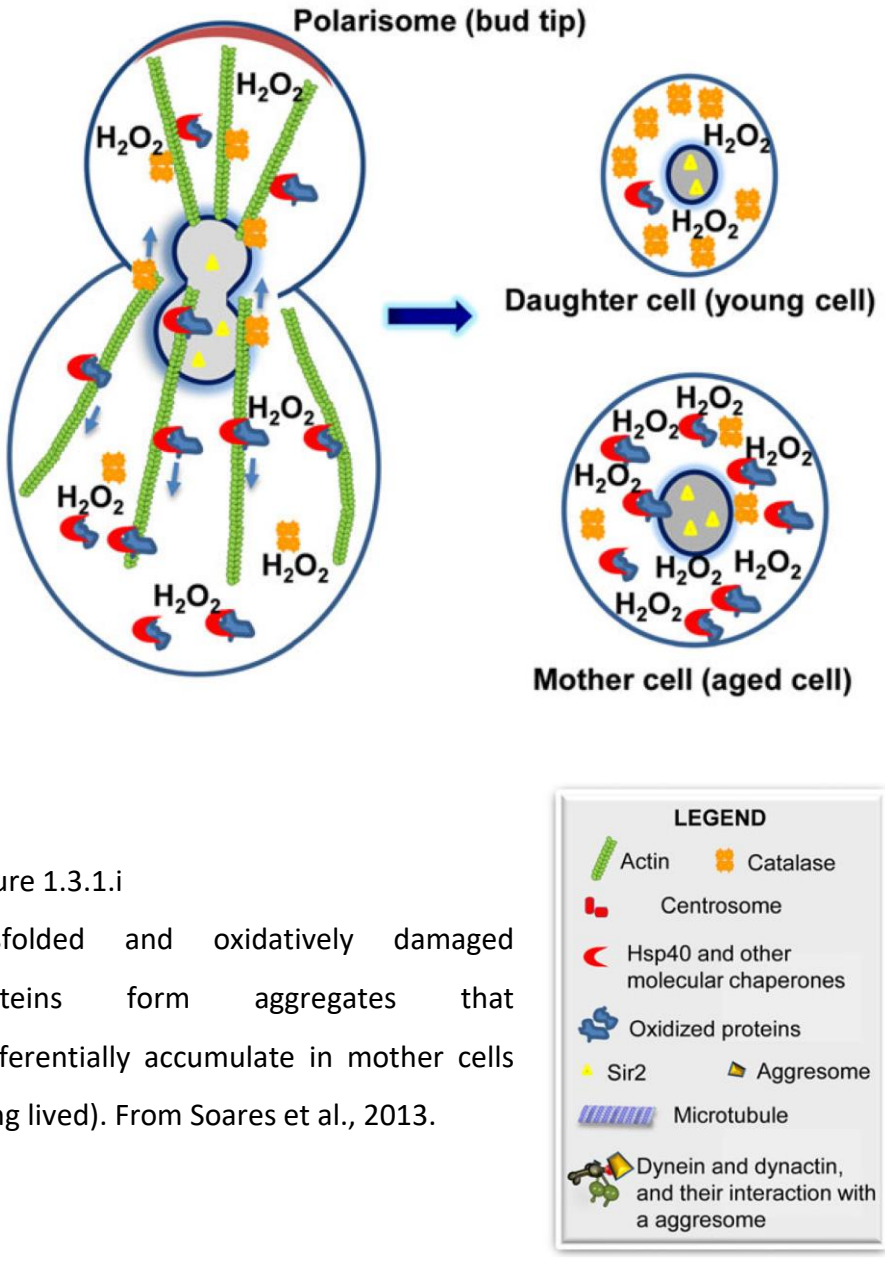


Figure 1.3.1.i

Misfolded and oxidatively damaged proteins form aggregates that preferentially accumulate in mother cells (long lived). From Soares et al., 2013.

1.3.1 The cell polarity of cancer

Alterations of epithelial tissue architecture underlie cancer development and progression by affecting cell–cell adhesion, cell matrix adhesion as well as protrusion formation, enabling tumor cell migration and invasion either via EMT or collective cell migration (Figure 1.3.1.i). Epithelial-to-mesenchymal transition that is associated with many carcinomas leads to loss of apical–basolateral polarity, loss of several epithelial markers, such as E-cadherin in adherens junctions, claudins, occludins, and junctional adhesion molecules. The loss of cell–cell junctions accompanied by the loss of primary cilia and deregulated proliferation due to the loss of contact inhibition results in invasiveness of cancer cells and metastasis to distal anatomical sites. Sometimes cancer cells prefer collective cell migration, migrating as group of cells without losing contacts.

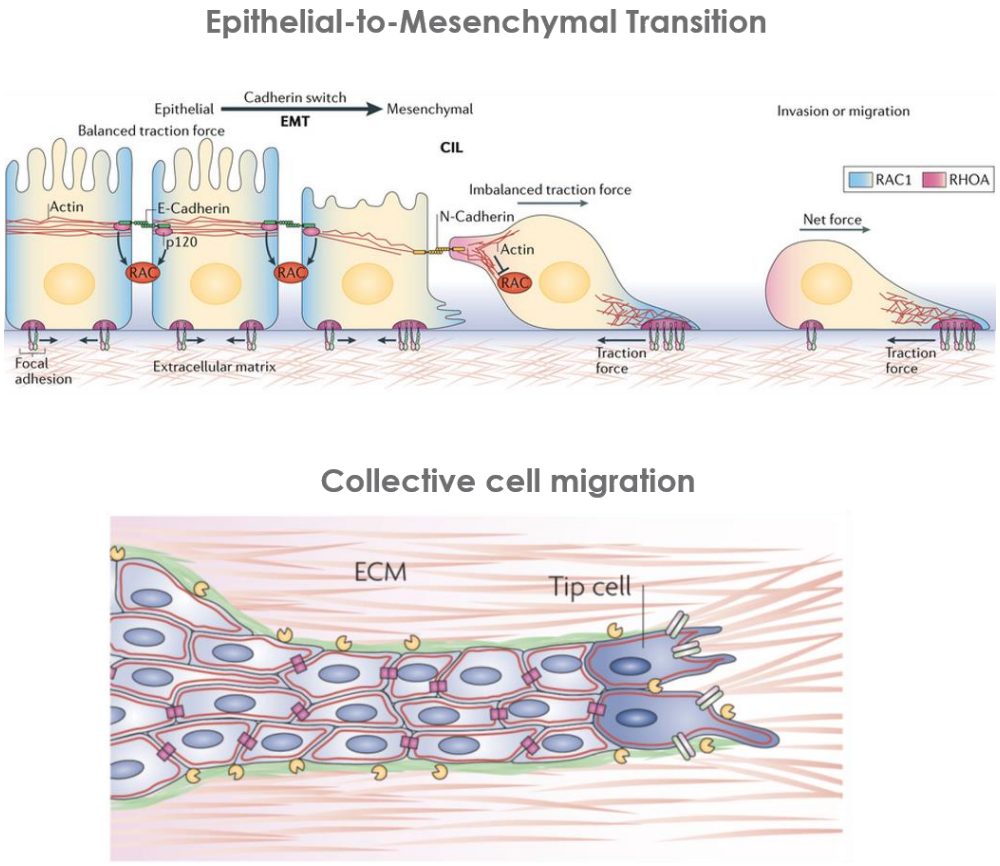


Figure 1.3.1.i

Polarity of migratory cells in cancer.

The cell polarity of cancer is also reflected in the deregulation of spindle orientation either at tissue or cellular level that could result in the appearance of cancer stem cells. More specifically, spindle disorientation in the plane of the tissue during symmetric cell divisions within a tissue could lead to tumor formation in cooperation with oncogenic mutations. Whilst, spindle disorientation during asymmetric cell divisions results in symmetric segregation of cell fate determinants, enabling the expansion of stem cell pool and tumor formation. (Figure 1.3.1.ii)

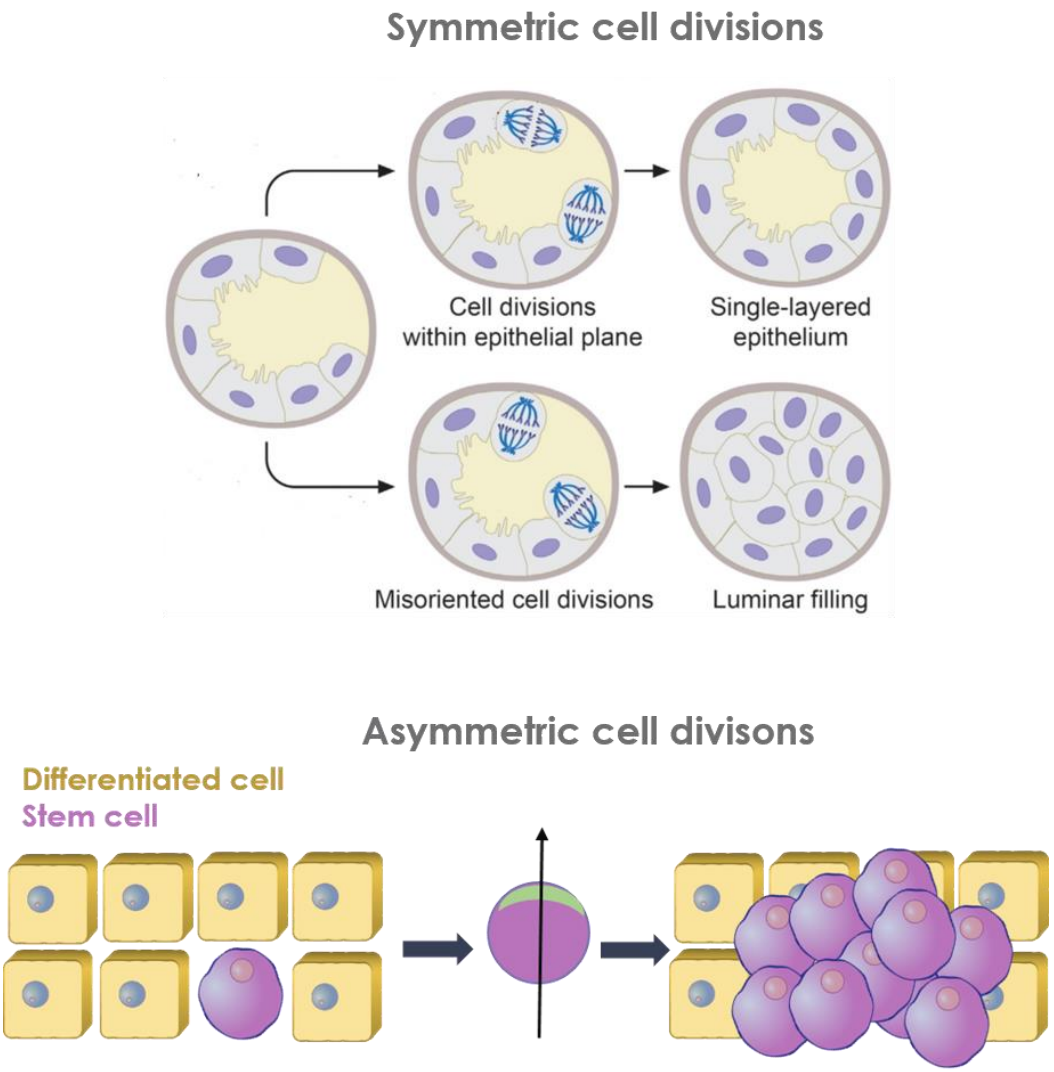


Figure 1.3.1.ii

Spindle disorientation at tissue and cellular level could lead to tumor formation through symmetric and asymmetric cell divisions respectively.

In general, tumor metastasis is a dynamic process, in which cancer cells acquire different phenotypes and display different types of cell polarity during their trip from the primary tumor mass till the new sites throughout the body where they found new colonies.

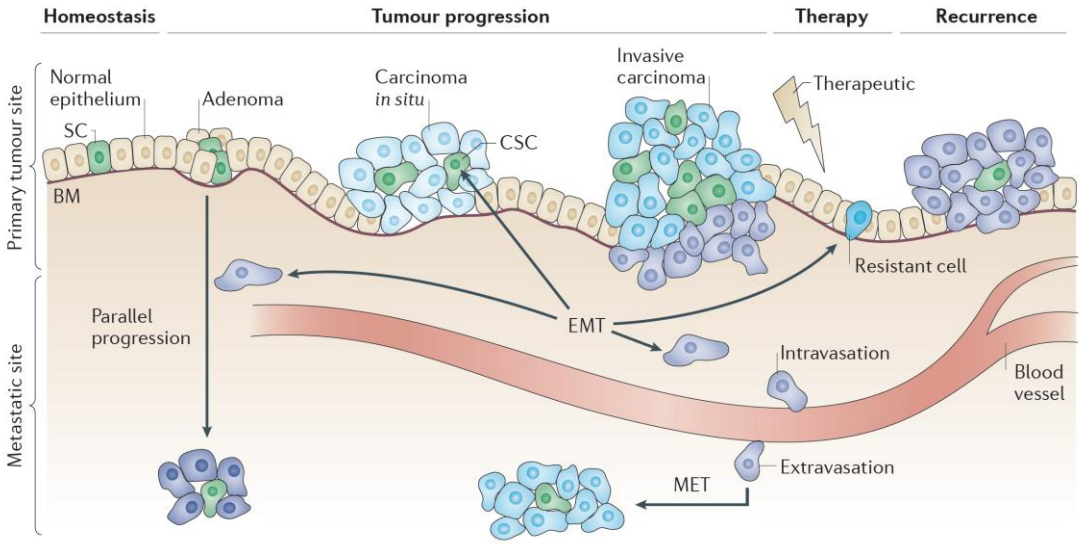


Figure 1.3.1.iii

Cancer cells metastasize invading the surrounding tissue.

From De Craene and Berx, 2013.

1.3.2 Cell polarity in breast cancer formation

Many dysregulated biological processes and signalling pathways observed in breast cancer progression mimic those observed during normal mammary gland development and tissue remodeling. Numerous of point mutations, translocations, amplifications and deletions are involved to breast cancer development. Cancer-related mutations affect several signaling pathways and processes, resulting to the loss of epithelial cell polarity and tissue architecture, cell hyperproliferation and elevated cell invasiveness which they are frequent features of malignant tumors. Loss of epithelial apical-basal polarity occurs in the early stages of tumor progression in breast tissue. The transition from epithelial to migratory polarity is the hallmark of cancer progression to an invasive and metastatic disease. Defects in polarity are induced directly by mutations in polarity proteins, but also indirectly by changes in the

expression of specific microRNAs and altered transcriptional programs that result in cellular differentiation from epithelial to more mesenchymal phenotype. Breast cancer develops through a multi-step process from benign epithelial atypia and atypical ductal hyperplasia (ADH), to malignant ductal carcinoma in situ (DCIS), or more rarely lobular carcinoma in situ (LCIS), and finally to invasive ductal carcinoma (IDC). All these stages of breast cancer progression acquire cell polarity transitions. For instance, the transition of the apico-basal polarity of luminal epithelial cells to cell apolarity because of the loss of cell adhesion molecules or the transition of apico-basal polarity to front-rear migratory cell polarity, in which the cells gain a mesenchymal phenotype.

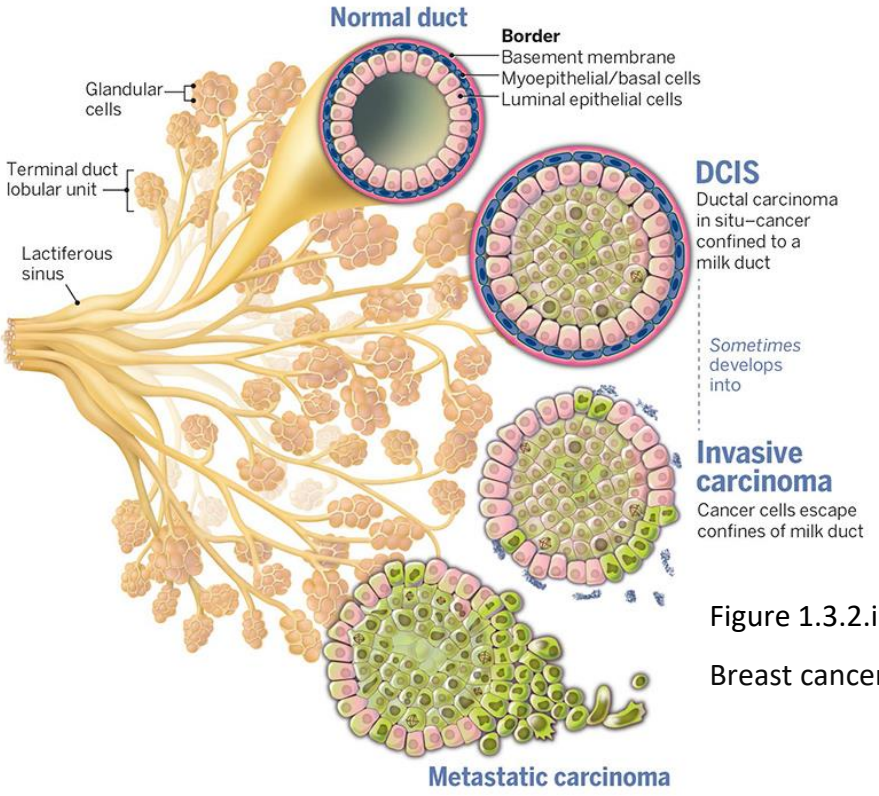


Figure 1.3.2.i

Breast cancer origin and evolution.

The detection of estrogen, progesterone and HER-2 receptors on the surface of the tumor cells constitute a significant prognostic factor in breast cancer. Based on gene expression profiling, breast tumors are classified into five intrinsic subtypes luminal A, luminal B, HER2 overexpression, triple negative/basal-like and normal-like. Luminal A breast cancer is hormone-receptor positive (estrogen-receptor and/or progesterone-

receptor positive), HER2 negative, with low levels of the protein Ki-67 and is characterized by the best prognosis. Luminal B breast cancer is hormone-receptor positive (estrogen-receptor and/or progesterone-receptor positive), and either HER2 positive or HER2 negative, with high levels of Ki-67 and slightly worse prognosis compared with Luminal A. Triple negative/basal like breast cancer is hormone-receptor negative (estrogen-receptor and/or progesterone-receptor negative) and HER2 negative. HER2 overexpression breast cancer is hormone-receptor negative (estrogen-receptor and/or progesterone-receptor negative) and HER2 positive. Normal-like breast cancer is similar to Luminal A breast cancer but its prognosis is slightly worse. (Dai et al., 2015)

Furthermore, new subtypes have been emerged based on signaling pathway signatures. The characterization of the activation status of several signaling pathways in a breast tumor provides the advantage of individualized therapies, targeting specifically the oncogenic activated signaling pathway.

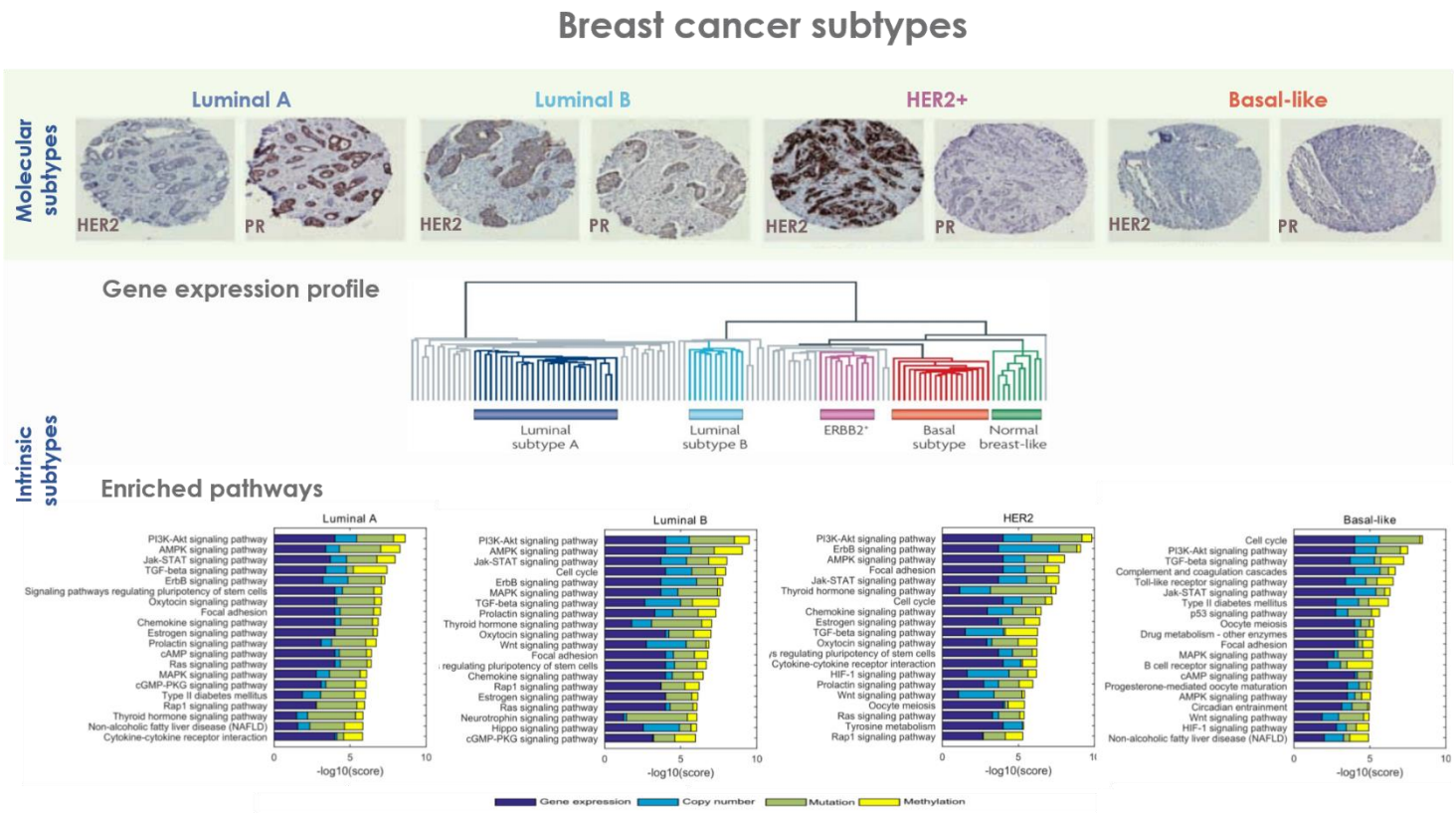


Figure 1.3.2.ii

Molecular and intrinsic subtypes of breast cancer.

1.3.3 Polarity proteins in cancer

Polarity proteins contribute significantly to the initiation and progression of cancer. Cell polarity is abolished in malignant epithelial tumors and disruptions in polarity genes can lead to epithelial tumors in the fly and in mammals (Humbert et al., 2003, Bilder, 2004, Hezel and Bardeesy, 2008, Humbert et al., 2008, Januschke and Gonzalez, 2008). In Drosophila, perturbed segregation of cell fate and polarity determinants leads to the expansion of the stem cell pool, which in turn leads to tumor formation.

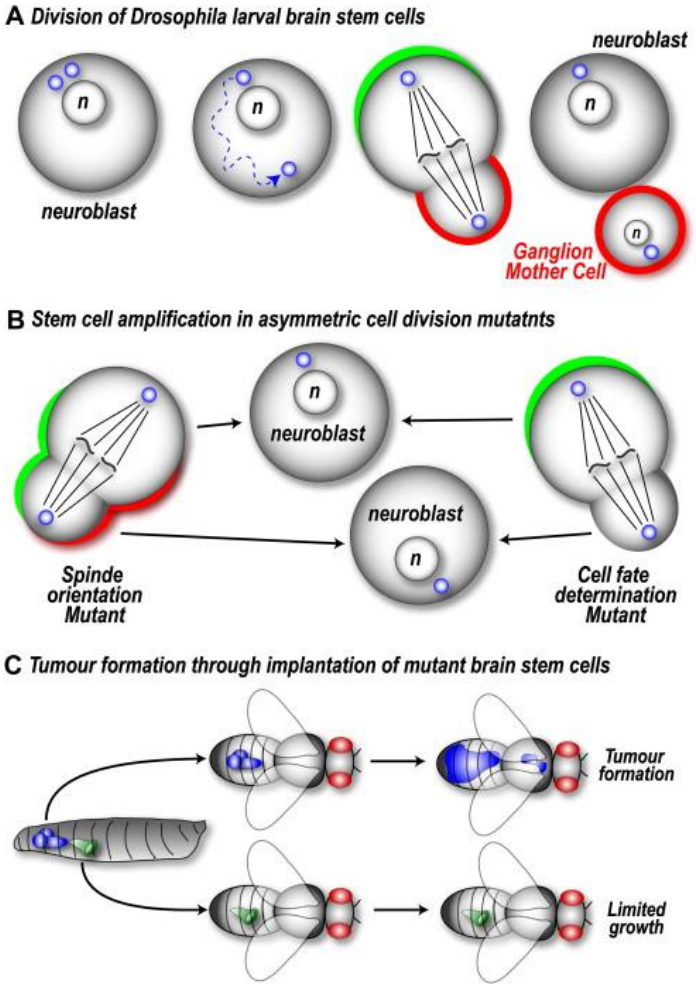


Figure 1.3.3.i

Perturbations in segregation of polarity and fate determinants results in tumor formation in Drosophila.

Alterations of polarity gene structure or regulation that leads to abnormal protein levels or subcellular localization due to various point mutations or gene amplification

have been observed in several cases of cancer (Vaira et al., 2011). In breast cancer, Par3 is downregulated compared to normal tissue, while its membrane localization is also decreased in metastatic cancer cells compared to their primary cancer cells (McCaffrey et al., 2012; Xue et al., 2013). aPKCι is upregulated in basal cell skin cancer and play a key role in hedgehog signaling activation, which mediates a positive feedback loop resulting in the transcriptional activation of aPKCι and promoting cell proliferation (Atwood et al., 2013).

Members of the Scribble complex, Scribble, Discs Large (Dlg) and Lethal Giant Larvae (Lgl), have been identified in *Drosophila melanogaster* as neoplastic tumor suppressors, with loss of function disrupting apical basal polarity and junctional integrity, and inducing inappropriate proliferation and tissue overgrowth (Bilder and Perrimon, 2000; Mechler et al., 1985; Woods and Bryant, 1991).

1.3.3.1 Scrib in cancer

Scrib was identified in a Drosophila screen for mutations that cooperate with activated Ras to promote tumour progression (Pagliarini and Xu, 2003). Loss of Scribble or the Dlg and Lgl, induce tumourigenic growth and promote spontaneous invasion and metastasis in the presence of the activated oncogenes Ras, Raf or Notch (Brumby and Richardson, 2003). In hematopoietic cells, genetic inactivation of Lgl1 leads to the expansion of stem cell pool that potentially could serve as a cofactor in cancer formation.

Scrib-defecient (*Scrib*-/-) mice are embryonic lethal (Murdoch et al., 2003, Pearson et al., 2011). *Scrib* heterozygosity is proven to be sufficient to cause focal prostate hyperplasia, whereas biallelic loss within prostate epithelium results to disease progression to low-grade prostate intraepithelial neoplasia through upregulation of the MAPK cascade (Pearson et al., 2011). Loss of Scrib function causes disruption of apical-basal polarity and junctional integrity and inappropriate proliferation, resulting in tissue overgrowth (Martin-Bemonte and Perez-Moreno, 2012).

Scrib constitute a target for ubiquitin-mediated degradation by the human papilloma virus (HPV) E6 proteins and E6AP protein ligase (Nakagawa and Huibregtse, 2000). HPV is frequently associated with cervical carcinomas, which often display decreased

Scribble expression (Nakagawa et al., 2004). Human T-cell leukemia virus type 1 (HTLV-1) Tax, the etiological agent of adult T cell leukemia, has been shown to target Scribble (Elsum et al., 2012, Javier and Rice, 2011, Okajima et al., 2008). Scrib loss and oncogenic K-ras cooperate to promote prostate cancer progression via ELK1 activation that is a direct target of the Ras/MAPK cascade (Pearson et al., 2011).

RasV12 transformed MCF-10A cultured in 3D cultures develop invasive protrusions and display loss of E-cadherin and polarity as well as loss of acinar organization (Dow et al., 2008). Elevated expression of Scrib blocks the RASV12 mediated effects, providing a compensatory mechanism of RASV12-mediated tumorigenic characteristics, whereas loss of Scrib promotes cell invasion through the activation of the MEK1/2-ERK1/2 signaling axis (Dow et al., 2008).

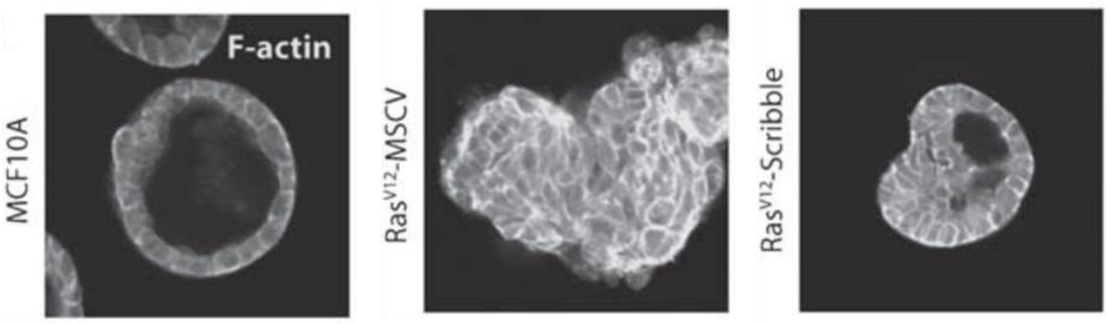


Figure 1.3.3.1.i

Scrib promotes anoikis in MCF10A RasV12 cells.

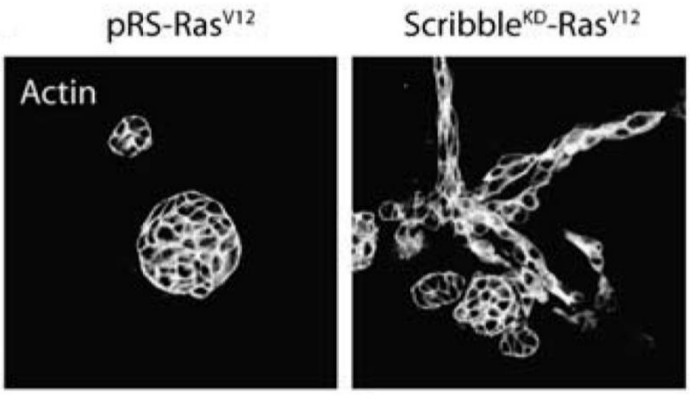


Figure 1.3.3.1.ii

Scrib loss promotes invasiveness properties, in MCF10A RasV12 cells cultures in Matrigel.

In mammary gland, loss of SCRIB activates RAS-MAPK signaling, and induces multilayering of the luminal epithelium and hyperbranching of ductal structures (Godde et al., 2014). In T47D breast-cancer-derived cell line, loss of SCRIB downregulates PRLR-JAK2-STAT5 signaling axis (Baker et al. 2016).

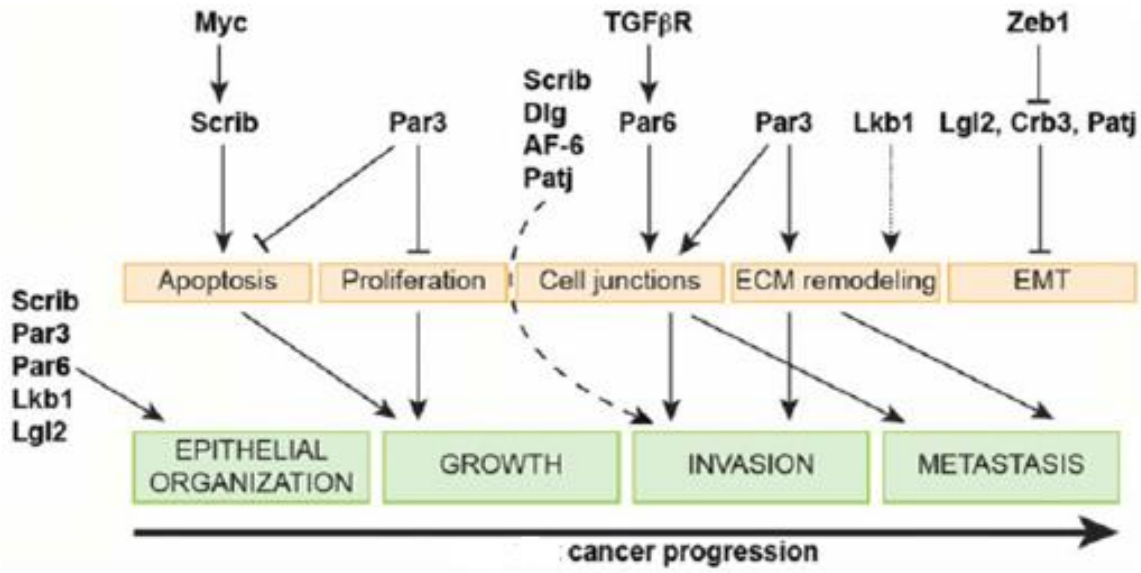


Figure 1.3.3.1.iii

Summary of Scrib module intervention in breast cancer development.

According to TCGA publically available genomic data SCRIB is amplified in several types of human cancer, such as breast cancer, ovarian cancer and prostate cancer (http://www.cbioportal.org/public-portal/) (Figure 1.3.3.1.v). Since the TCGA provides the genomic profiling and RNA sequencing data of thousands of samples representing all major cancer types, we assessed the expression of Scrib in these samples and generated their profiles. The expression levels of SCRIB from the TCGA tumor RNA sequencing data were compared among patient samples representing breast cancer subtypes.

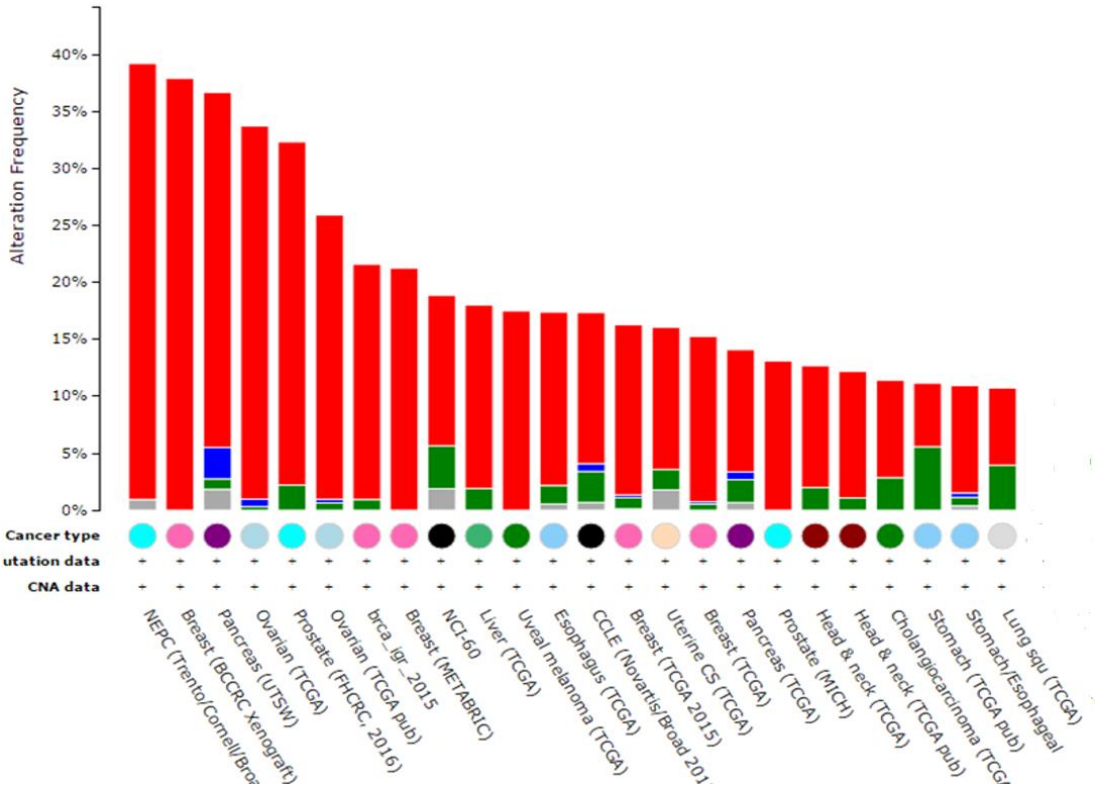


Figure 1.3.3.1.v

SCRIB is frequently amplified in many cancer types. DNA amplification (red), deletion (blue), and mutation (green), and multiple alterations (gray) are indicated.

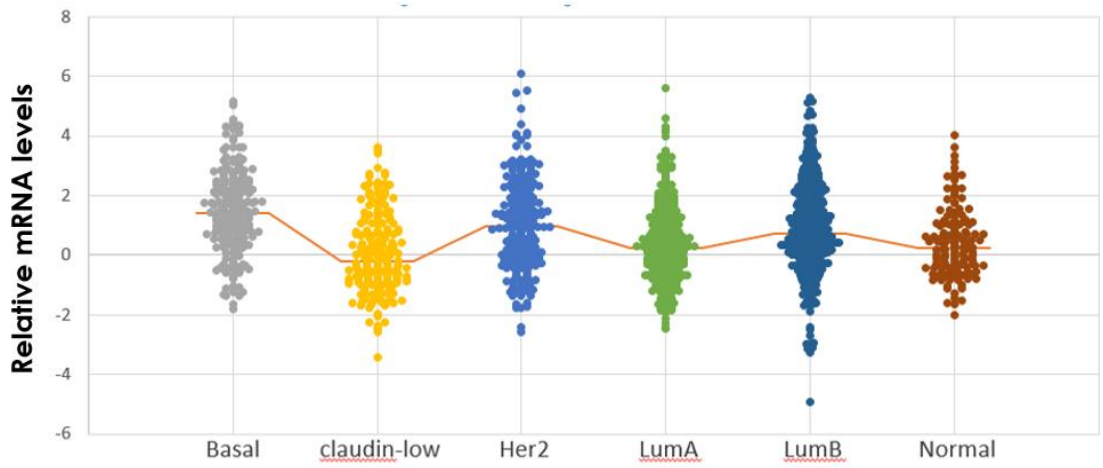


Figure 1.3.3.1.vi

Relative expression of *Scrib* mRNA from different breast cancer subtypes.

Several SCRIB truncation and missense mutations have been investigated according to TCGA genomic data analysis of patient samples, in several types of human cancer. It is worth mentioning that truncations encoding the N-terminal LRR domain of SCRIB with loss of the C-terminal part of the protein have been reported in breast invasive ductal carcinoma, in stomach adenocarcinoma, as well as in uterine endometroid carcinoma.

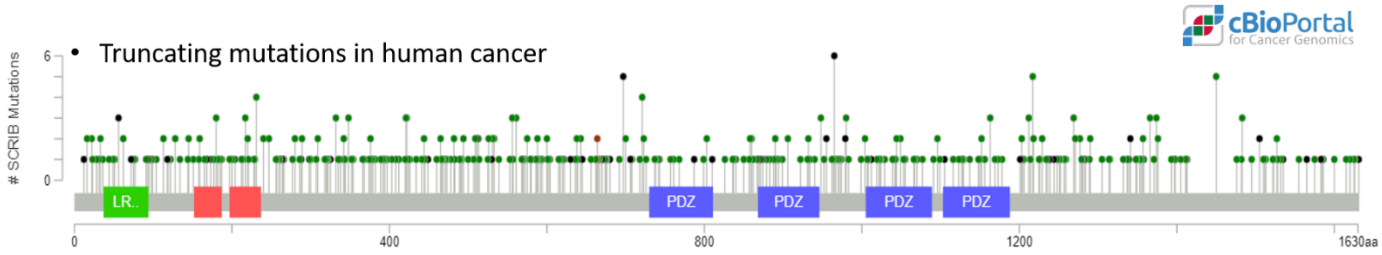


Figure 1.3.3.1.v

Schematic of mutation's position in SCRIB gene found in human cancer.

Scrib re-localize from lateral membranes to the cytosol in epithelial cancers. Mislocalization of Scrib has been reported in a variety of epithelial cancers. In the atypical dysplastic glands of adenomatous polyp, the pattern of Scrib expression is diffused to the cytoplasm, apart from the basolateral region where it localizes in normal glandular epithelia. In colon adenocarcinomas, Scrib protein lie at the apical domain of the cells as well as at sites of cell contact, whereas in poorly differentiated carcinoma samples (histological grade 3) Scrib was expressed focally, with a severely altered distribution at the periphery of the cells (Gardiol et al., 2006). Expression of the epithelial-to-mesenchymal transcription factor Snail in benign epithelial cells leads to Scrib mislocalization from the plasma membrane to the cytosol, mimicking the mislocalization observed in aggressive cancers. To do so, Snail represses the expression of many protein acyl transferases while promotes the expression and activity of protein acyl thioesterase 2 (APT2), abrogating Scrib palmitoylation-mediated membrane association (Hernardez et al., 2017).

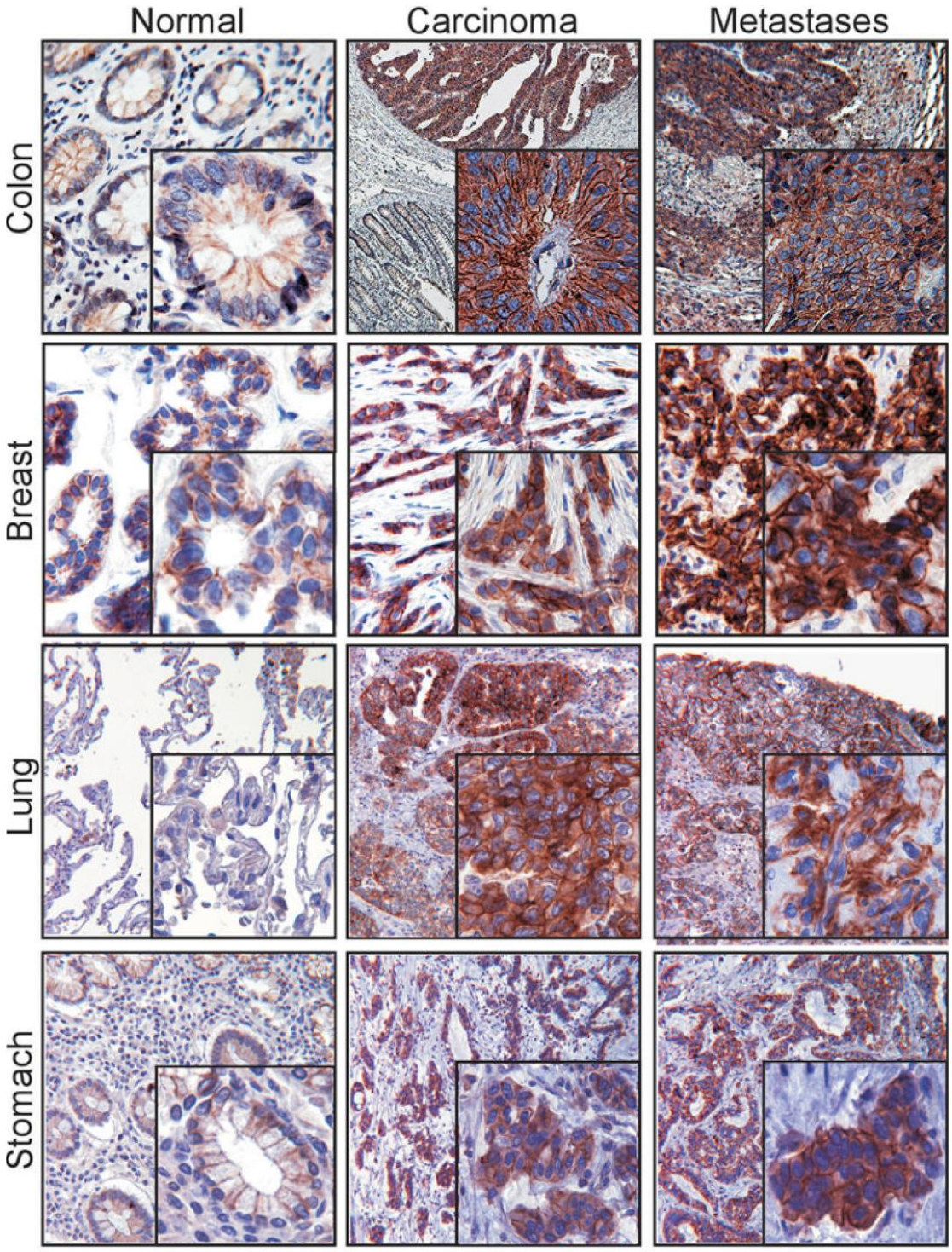


Figure 1.3.3.1.vi

Human histological samples of Scrib expression.

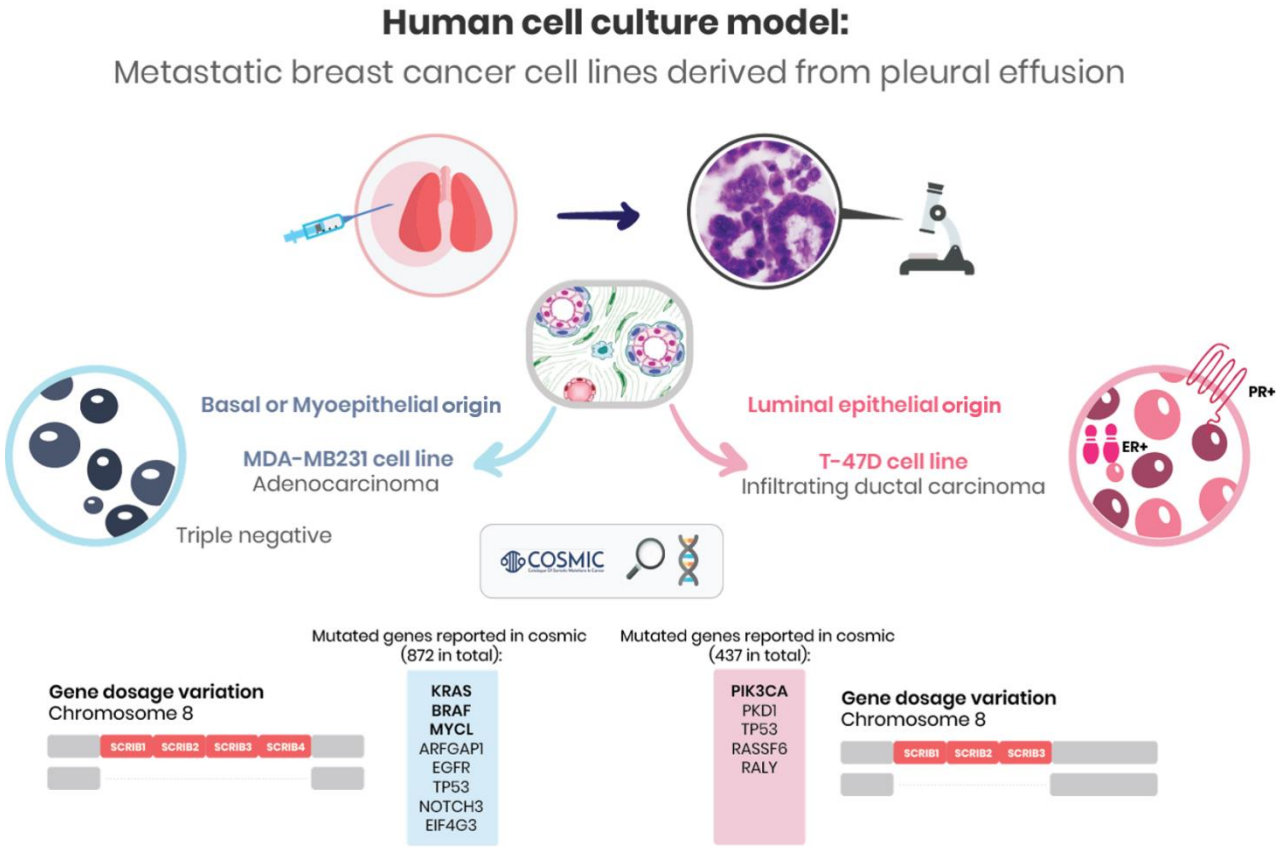
From Viara et al. 2011

2. Study objectives

Our aim is to investigate the role of polarity proteins in tumor progression and metastasis. Various functions of polarity proteins are well-characterized in *Drosophila,* but there is still less understanding of their role in mammals and in different cell contexts. Our study focuses on the characterization of the molecular mechanisms that interfere with normal cell polarity, the identification of molecular interactors of polarity complexes and the determination of activated oncogenic pathways that co-operate with polarity proteins in order to achieve malignant transformation and abnormal invasive and migratory (front-rear polarity) behavior. We are particularly interested in the role of the polarity protein SCRIB in breast cancer formation. Cancers that originate from epithelial tissues as well as those that come from other tissues often utilize similar molecular mechanisms to become aggressive. SCRIB is a multi-tasking scaffold protein whose proper localization within the cell is an important feature for its correct function. SCRIB consists of an N-terminal region with 16-leucine-rich repeats (LRR) that is responsible for its membrane targeting, following 4 PDZ domains that interact with proteins such as ZO-2, APC, βPIX, zyxin family and a C-terminal region that contains three spectrin binding motifs. According to publicly available cancer genomics resources (TCGA, CBioPortal) and bioinformatic analysis that have been conducted, the most common alterations of Scrib in human cancer are gene amplification and increased expression. Several studies support the involvement of Scrib in human cancer, showing that: 1) Loss of Scrib cooperates with myc oncogenes to block apoptosis and promote cell transformation and breast cancer formation. 2) Loss of Scribble cooperates with activating Ras mutations to promote tumorigenesis and tumor progression. 3) Overexpression of Scrib inhibits expression of ZEB-1 and promotes the expression of tight junction proteins. 4) Scrib controls the localization and activity of Cdc42 and Rac1 at the leading edge of migrating cells. Therefore, Scrib has been linked to multiple steps of cancer development and progression.

We used the MDA-MB231 (ER-, PR-, HER2-) and T-47D cell lines, as a model for breast cancer. By employing overexpression approaches, we studied the functions of the polarity protein SCRIB as well as the functions of a truncated Scrib in cancer growth

and metastasis. To achieve this, we generated stable cell lines where we overexpress the truncated Scrib, using doxycycline-dependent inducible RFP-tagged $SCRIB_{LRR}$ expression vectors. The invasiveness of cancer cells before and after the polarity gene overexpression was studied *in vitro* using a fluorescent substrate (FITC) that mimics the extracellular matrix and was monitored by loss of matrix fluorescence. Alterations in gene expression profiles and intracellular signaling, upon overexpression of the genes of interest were studied by RNA sequencing, qPCR, Western blotting and immunofluorescence. Flow cytometry was used to detect apoptosis and Epithelial-Mesenchymal Transition. As Scrib interfere with several signaling pathways, we tried to determine which signaling axis crucial for cell functions are affected, using phosphospecific antibodies, pharmacological inhibitors and siRNA-based approaches.



3. Materials and methods

3.1 Cell culture and cell treatments

MDA-M231 and T-47D human breast cancer cell lines as well as U-87 MG glioma cell line were purchased from the ATCC cell bank. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, InvitrogenTM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and antibiotics 100 units/ml penicillin and 100 µg/ml streptomycin (Life technologies). Cells were cultured in a humified incubator at 37 °C under 5% CO2. Before reaching confluence, cells were dissociated by adding 1X Trypsin-EDTA (Gibco) and then split into new dishes for passages or used for experiments.

Tumorspheres were grown in DMEM-F12 1:1 (Gibco) medium supplemented with B27 (1:50), bFGF (20ng/ml), EGF (20ng/ml) and 0.2% methylcellulose, in ultralow attachment plates. For the generation of doxycycline inducible cell lines, first an expressing pLenti CMV rtTA3 Blast (w756-1) plasmid (Addgene #26429) was introduced into recipient cells and the positive cells were selected with blasticidin (5µg/ml). Next, the dox inducible toScrib94-494xRFP or toScribFLxRFP was introduced into recipient cells and the positive cells were selected with puromycin (5µg/ml).

All treatment media were supplemented with 10% heat-inactivated FBS previously dialyzed through 3500 MWCO dialysis tubing. Cells were cultured without or in the presence of 1µg/ml doxycycline (dissolved in distilled water) for different time points (2, 4, 12, 24, 48 or 72 h). Doxycycline was added to 80% confluent cells. Media containing the drug was refreshed every 48 hours.

For glucose starvation experiments, DMEM glucose-free (Gibco) has been supplemented with dialyzed FBS and the antibiotics listed above. At 80% confluence, culture medium was removed, cells were washed once with glucose-free starvation medium, and then the cells were cultured in glucose-free starvation medium for 24h. For amino-acid starvation, -aa medium was prepared according to the Gibco recipe for high-glucose DMEM, omitting the amino acids, and filtered through a 0.22-µm filter device before use. Normal culture medium was replaced with –aa medium for 1,5 h.

For serum starvation experiments, the culture medium was removed, cells were washed once with media lacking FBS and then cultured in media lacking FBS for 16 h. Chemical and pharmacological treatments using Akt inhibitor VIII (#124018, Calbiochem), TC ASK10 (#4825, Tocris), PRI-724 (#S8262, Selleckchem), SP600125 (#S1460, Selleckchem), mTOR inhibitor Torin1 (#Cay10997, Cayman Chemical), Rapamycin (#553210, Sigma) and CoCl2 (#60818, Sigma) were performed by adding the drugs to the medium at concentrations indicated in the results chapter. Treatment for hypoxia induction was implemented in the presence of 150µM CoCl2 for 24h to culture media. Transfections were performed with Lipofectamine RNAiMax (Life Technologies), according to the manufacturer's instructions.

3.2 Antibodies

Antibodies against phospho-S6K(T389) (#9205), S6K (#9202), phospho-4E-BP1(T37/46) (#9459), 4E-BP1 (#9452), phospho-Akt(S473) (#9271), phospho-Akt(T308) (#9275), Akt (#9272), phospho-TSC2(T1462) (#3611), phospho-TSC2(S1387) (#5584), phospho-TSC2(S1254) (#3616), phospho-TSC2(S939) (#3615), TSC2 (#4308), mTOR (#2983), phospho-GSK3β(Ser9) (#5558), GSK3β (#12456), phospho-p38(T180/Y182) (#9216), p38 (#9212), phospho-ERK1/2(T202/Y204) (#4370), ERK1/2 (#4695), phospho-Erk5(T218/ Y220) (#3371), Erk5 (#3372), cleaved caspase-3 (#9661S), RagC (#5466), phospho-c-Jun (#3270), c-myc (#5605), Notch1 (#3608), Notch2 (#5732) and Notch3 (#5276) proteins were purchased from Cell Signaling Technology. HIF1a antibody (GTX127309) was obtained from GeneTex. Antibodies against Scrib (C-20) (sc-11049), phospho-JNK (G-7) (sc-6254), JNK (D-2) (sc-7345), β-catenin (E-5) (sc-7963) and β -ACTIN (sc-47778) proteins were purchased from Santa Cruz. NUMB (ab14140) antibody was purchased from abcam. Anti-KillerRed (AB961) antibody was purchased from Evrogen. RCFP polyclonal pan antibody (#632475) was obtained from clontech. The mouse LAMP2 (ABL-93) antibody was purchased from Developmental Studies Hybridoma Bank. A monoclonal antibody recognizing human and mouse α-tubulin (#T9026) was purchased from Sigma. All antibodies were used in 1:1000 dilution for western blotting.

3.3 Plasmid constructs

For the Scrib94-494 RFP tagged expression vector, SCRIB94-494 was obtained from the pLK01 plasmid (addgene, #37252) digested with SalI and StuI enzymes and cloned into JRed-C2 vector (addgene, #54788) in the SmaI/SalI sites. JRed-C2_SCRIB94-494 digested with BamHI and NheI restriction enzymes and cloned into pMA2780 vector digested with XbaI and BamHI enzymes. ScribFL RFP tagged vector, SCRIBFL_mRed was obtained from pLK45_mRed plasmid (addgene) digested with NheI, HpaI restriction enzymes and cloned into pMA2780 (addgene) digested with XbaI, SmaI enzymes. For PON RFP tagged expression vector, PON (aa450-623) was amplified from *Drosophila* cDNA with primers designed containing NheI and SalI restriction enzyme overhangs in the sites of Lentiviral mRedpL vector. Lentiviral mRedpL vector was created from pDsRed-Monomer-C1 (addgene) digested with EcoRI enzyme and cloned into NUMB4-eGFP-M10K plasmid (addgene, #37805) digested with SphI and BglII restriction enzymes. Amplified PON (aa450-623) cloned into lentiviral vector mRedpL digested with SalI restriction enzyme. All constructs were sequenced to confirm their integrity.

3.4 Virus production

For lentivirus production, the lentiviral constructs and second-generation packaging plasmids pMD2.G (Addgene, #12259) and psPAX2 (Addgene, #12260) were co-transfected into HEK293T cells. Lentivirus-containing supernatant was collected 48 and 72 h after transfection and filtered (0.45 mm) before use. Transfections were performed using the calcium phosphate DNA precipitation method.

3.5 Western blot

Total cell proteins were prepared by lysing the cells in-well with ice-cold RIPA lysis buffer (50mM Tris-HCl pH8, 150mM NaCl, 2mM $MgCl_2$, 2mM $CaCl_2$, 0,5% sodium deoxycholate, 1% NP40, 0,1% SDS, 10% Glycerol, 1x PhosSTOP phosphatase inhibitors and 1x Complete protease inhibitors), for 10 min on ice and then for 20min on a rotating wheel at 4^0C. The lysates were centrifuged (10min, 16000rpm, 4^0C) and total protein was estimated by Bradford assay. SDS loading buffer was added to the soluble

protein fraction before boiling. 30µg of samples were separated on Tris-Glycine gel and transferred to PVDF membrane (Johnson 0,45µm). Membranes were blocked in TBS-T 5% BSA for 30 min at room temperature followed by incubation of primary antibody in TBS-T 3% BSA overnight at 4°C. They were then washed three times with TBS-T for 10 min and incubated with horseradishperoxidase-conjugated secondary antibodies in TBS-T 5% milk at RT for 1h. Blots were developed with the ECL system (Thermo Scientific).

3.6 RNA-sequencing analysis

Total RNA was isolated from SCRIB94-494 OE and control cells using TRIzol (Invitrogen). The RNA-sequencing was performed by the Illumina NGS platform. The gene expression levels were estimated as RPKM values. Genes with low expression (average RPKM < 0.5) were filtered out, while genes were considered differentially expressed if they exhibited a Benjamini and Hochberg–adjusted P value (FDR) <1% and a mean fold change >2.

3.7 Quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. 2µg RNA was reversely transcribed to cDNA by M-MuLV Reverse Transcriptase (Biolabs) supplemented with RNase inhibitor (Biolabs) according to the manufacturer's instructions. Quantitative real-time PCR was carried out using SYBR Green I (Invitrogen). Relative mRNA expression levels are calculated after normalization against β-Actin levels.

3.8 Transfections with siRNA

Cells were plated and transiently transfected with single-stranded RNA (siRNA) oligonucleotides. Predesigned siRNA for human RRAGA, RRAGB, RRAGC, RRAGD, RHEB, CTNNB1, MYC and as negative control a not-targeting siRNA (siNT) was purchased from Sigma. Cell lines were transfected with 0.05µM siRNAs using RNAimax (invitrogen) according to manufacture instructions. Each oligonucleotide and 2.5µl Lipofectamine RNAimax (Invitrogen) was diluted into 500 µl of OPTIMEM-I Reduced Serum Medium (Gibco, Invitrogen).

3.9 Matrix degradation assay

Coverslip cleaning: 18-mm glass coverslips (Marienfeld) were incubated overnight in HCl 1N, extensively washed in dH2O, incubated in 96% EtOH for 30 minutes, again washed in dH2O, incubated 30 minutes in acetone, washed again, let drying and sterilized in autoclave.

Coverslip coating with gelatin and cell plating: In a 12-well plates, one sterilized coverslip per well was coated with 1ml/well of 5µg/ml poly-L-lysine (Invitrogen) in deionized water for 20 minutes at room temperature. The poly-L-lysine was then removed, and the slides rinsed three times with 1mL/well PBS. Thereafter, 1ml of 0.5% glutaraldehyde in PBS was added per well for 10 minutes at room temperature to activate the poly-L-lysine surface for further protein attachment. In order to remove the glutaraldehyde, each well was again rinsed three times with 1ml of PBS. After that, 80µL of gelatin from pig skin fluorescein gelatin (G13187, Invitrogen) was added for 10 minutes at RT, followed by three washes with PBS. Additionally, a wash with culture media containing FBS as a reducing agent was performed. 20000 cells/well were plated on the gelatin-coated coverslips that are inserted into the wells. Cells were seeded and allowed to attach and grow.

3.10 Immunofluorescence

The cells were fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were washed 3 times with 0.05% Tween-PBS and permeabilized in 0.5% NP40-PBS (Roche) for 5 min at room temperature followed by three rinses. Thereafter, samples were blocked with PBS 1% BSA for 1h. Then, the samples were incubated with primary antibodies diluted in PBS with 3% bovine serum albumin (BSA, Sigma) for 1 h at room temperature. After washing 3 times with PBS, the samples were incubated with fluorophore-conjugated secondary antibodies diluted in PBS with 3% BSA at room temperature for 1h. Then the cells were washed for another 3 times. Finally, cells were mounted with ProLong Diamond Antifade Mountant with DAPI (ThermoFisher, #P36962).

Confocal microscopy was carried out on a Laser-scanning confocal Leica SP8-X**.** For matrix degradation assay, cells images (10 fields per condition) were subsequently

captured at a ZEISS Apotome microscope using a 40x objective, as well as at Operetta High-Content Imaging System.

3.11 Quantification of matrix degradation

The fluorescein gelatin images were analyzed using ImageJ (NIH) software. An identical thresholding was used for all images and for all conditions of the same experiment. After thresholding, the number of black pixels per each field was counted and divided by the number of nuclei.

3.12 Cell cycle analysis

For cell cycle analysis, 100.000 cells from each sample were trypsinized, washed with PBS, treated with RNAse A for 30 min at 37^{O}C and stained with propidium iodide (PI - Sigma) according to the manufacturer's protocol. The analysis was conducted using a FACS Calibur analyzer. The cell cycle profile was further analyzed using the ModFit LT software.

3.13 Apoptosis assay

Cells were stained using the Annexin V-FITC Early apoptosis detection kit (cell signaling, #6592) according to the manufacturer's instructions. Stained cells were diluted in 1X Annexin V-binding buffer. Suspended cells were used to perform flow cytometry. The analysis was conducted using a FACS Calibur analyzer. The cell cycle profile was further analyzed using the ModFit LT software.

3.14 Protein immunoprecipitation

IP of in vivo interacting protein complexes was performed using stable cell lines or HEK 293T cell extracts prepared by RIPA lysis buffer. 200µg of protein extracts were incubated with primary antibody overnight at 4°C. Next, 20µl of protein G beads were added to each sample after washing with IP buffer (25mM Tris-HCl pH 7.6, 150mM NaCl), and reactions were incubated at 4°C for 3 additional hours. Non-specific proteins were washed away three times with NETN buffer (10mM Tris-HCl pH 8.0, 250mM NaCl, 5mM EDTA, 0.5% N-40, 1mM PMSF). SDS sample buffer was added and

the samples were boiled prior to SDS -PAGE analysis. Input lanes represent 10% of the lysate used for the immunoprecipitation.

3.15 Xenograft models

Immunocompromised mice received subcutaneously 100µl of cell suspension in 1:1 Matrigel (Corning): 1000 cells per animal in PBS. Tumor volume was assessed by measuring tumor length and width using digital calipers twice per week. Mice were sacrificed on 5^{th} week after the injection. Metastatic colonies, circulating tumor cells (CTCs), as well as part of the primary tumor mass were isolated and subjected to 2D cell culture. If necessary fresh collagenase (thermofisher) added to dissociate the cells from tissue. Metastatic sites of xenograft experiment were viewed under a Leica MZ9.5 fluorescent stereoscope equipped with mercury lamp power supply and GFP filter set.

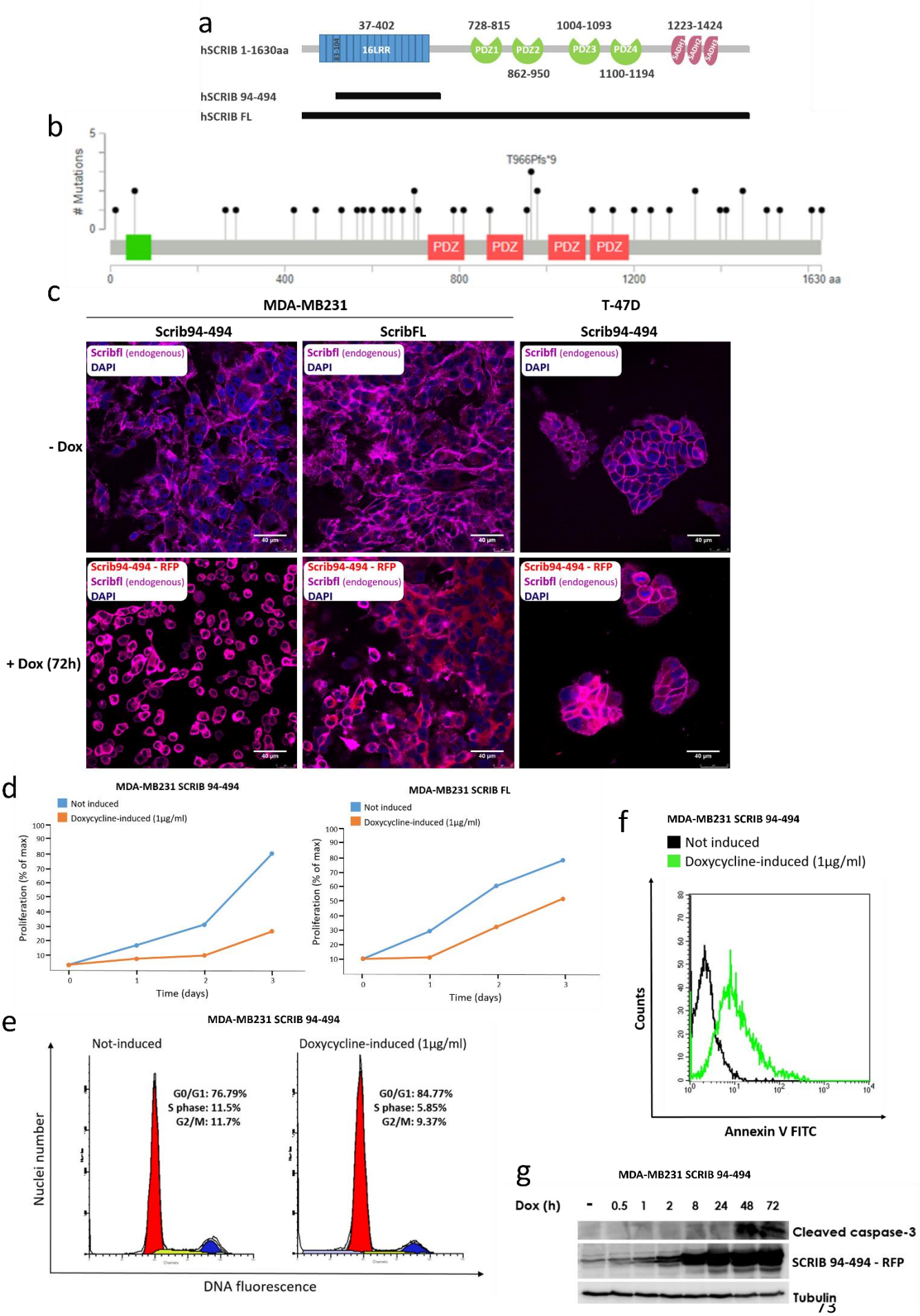
4. Results

4.1 Overexpression of the truncated Scrib 94-494 drives MDA-MB231 cells to apoptosis.

Dysregulation of oncogenic signaling pathways, such as Ras, has been proven to cooperate with SCRIB protein, leading to tumor formation and progression (Dow et al., 2008). We analyzed genomic data from Cancer Genome Atlas (TCGA) and deduced that in several cancers, including breast cancer, mutations in the sequence of Scrib lead to truncated forms of the protein (Fig. 1b). For instance, truncations encoding the N-terminal LRR domain of SCRIB with loss of the C-terminal part of the protein have been reported in breast invasive ductal carcinoma, in stomach adenocarcinoma, as well as in uterine endometroid carcinoma. Furthermore, Metodieva et al. showed that some breast cancers apply distinct Scrib exon usage pattern, which results in overexpression of conserved exons encoding the N-terminal LRR domain and loss of the exons encoding the C-terminus, rejecting the cell cycle checkpoint function of the C-terminal part of SCRIB.

To examine the role of a truncated Scrib mutant that contains the LRR domain of Scrib, we used human breast cancer derived cells in which SCRIB gene is amplified in one chromosome while it is deleted in the other one. We employed an inducible, tetracycline-on system to control the ectopic expression of Scrib94-494 – RFP in MDA-MB231, a highly aggressive triple negative breast cancer cell line. Following doxycycline treatment (1μg/ml), Scrib94-494 over-expressing cells demonstrated a rounder morphology with decreased cell-cell contacts as compared to un-induced controls that appeared elongated (Fig. 4.1c). To test if the phenotypic effect of truncated Scrib is cell context dependent, we decided to use the ductal carcinoma cell line T-47D with a Tet-On inducible expression system for controlled expression of the truncated Scrib. In this cell context, Scrib94-494 did not affect cell morphology and cell-cell contacts were preserved. Immunofluorescence staining using a Scrib polyclonal antibody, which recognizes the C-terminal part of the protein, revealed that endogenous Scrib is localized at the cell surface and inside the cell in MDA-MB231 Scrib94-494 un-induced cells, whereas in the presence of doxycycline we observed diffused cytoplasmic localization of endogenous Scrib colocalized with the ectopically

expressed truncated Scrib. In T-47D cells upon doxycycline stimulation endogenous Scrib did not exist only at the cell surface, as it is observed to the controls but it is also diffused to the cytoplasm colocalized with the ectopically expressed truncated Scrib (Fig. 4.1c). Additionally, in T-47D dividing cells, we found cytoplasmic dot-shaped structures positive for Scrib, indicating that association and dissociation of the polarity protein Scrib from the plasma membrane occurs during mitosis. Next, we observed the induction of Scrib 94-494 is accompanied by reduced cell counts (Fig. 1d). To determine whether Scrib full length exert a similar effect on cell morphology and proliferation rate, we generated an inducible tetracycline-on system to control the ectopic expression of ScribFL – RFP in MDA-MB231 cells and we performed a growth curve for that cells as well. We concluded that ScribFL results in a moderate reduction of proliferation rate (Fig. 1d). To pinpoint if that reduction in cell number is associated with cell cycle arrest, the Scrib 94-494 expressing cells and the un-induced controls were analyzed by flow cytometry for DNA content after propidium iodide staining. We found that Scrib 94-494 expressing cells undergo a G0/G1 cell cycle phase arrest 48h after doxycycline treatment (Fig. 1e). The function of Scrib 94-494 in apoptosis was assessed by a flow cytometry approach using FITC-AnnexinV staining and analysis of pro-caspase-3 proteolytic activation by Western blotting. Using flow cytometry to detect apoptosis, revealed that MDA-MB231 Scrib94-494 were positive for Annexin, V FITC, indicating the involvement of the LRR domain of Scrib in early apoptosis events. Western-blot analysis of whole cell lysates indicated that in the presence of doxycycline (1μg/ml) in the culture medium, the truncated Scrib is expressed within 2h and peaked at 24h following induction, whereas detection of Caspase-3 cleavage followed much later at 48h after treatment, implying the involvement of the truncated Scrib in caspase-mediated apoptosis (Fig. 1g). In contrast to MDA-MB231 Scrib94-494 cells, ectopic expression of Scrib94-494 in T-47D cells, did not lead the cells to apoptotic death. These results together indicate that the LRR domain of Scrib constitute a major motif that is responsible for Scrib-mediated cell cycle arrest as well as cell death induction, and its role in different types of cancer is context-dependent.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83-104
16LRR
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862-950
1100-1194
hSCRIB 94-494
hSCRIB FL
b
Mutations
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PDZ
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400
800
1200
1630 aa
c
MDA-MB231
T-47D
Scrib94-494
ScribFL
Scrib94-494
- Dox
+ Dox (72h)
Scribfl (endogenous)
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Scrib94-494 - RFP
d
MDA-MB231 SCRIB 94-494
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Not induced
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Not-induced
Doxycycline-induced (1µg/ml)
G0/G1: 76.79%
S phase: 11.5%
G2/M: 11.7%
G0/G1: 84.77%
S phase: 5.85%
G2/M: 9.37%
Nuclei number
DNA fluorescence
f
MDA-MB231 SCRIB 94-494
Not induced
Doxycycline-induced (1µg/ml)
Counts
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g
MDA-MB231 SCRIB 94-494
Dox (h)
- 0.5 1 2 8 24 48 72
Cleaved caspase-3
SCRIB 94-494 - RFP
Tubulin

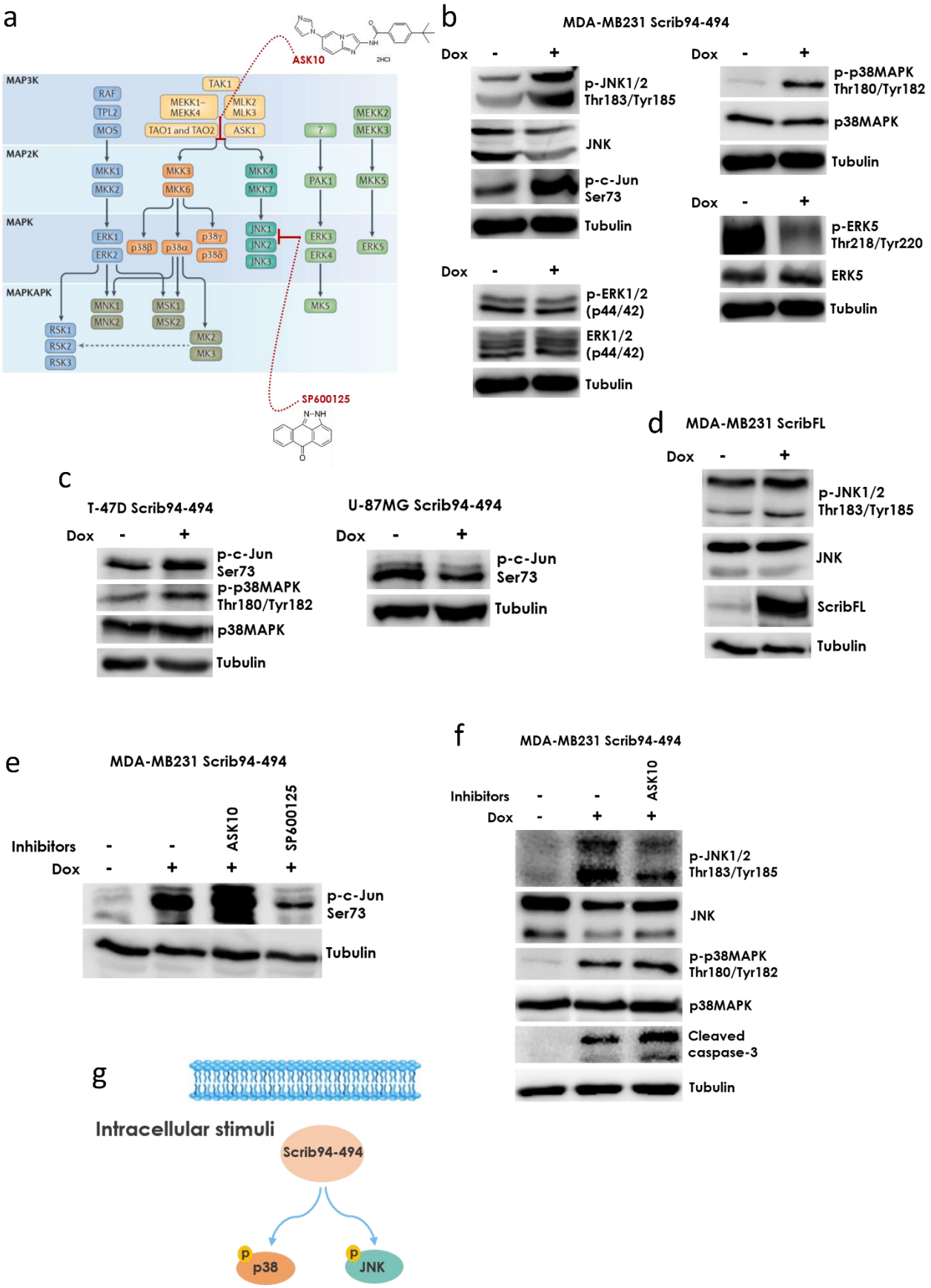
Figure 4.1. SCRIB 94-494 -mediated cell growth arrest and apoptosis in human breast cancer cell lines. a) Schematic of the SCRIB domains and the truncated SCRIB constructs used in this study. **b)** Truncating mutations in SCRIB, in human cancer. **c)** Representative images of endogenous Scrib and ectopically expressed truncated Scrib levels of MDA-MB231 Scrib94-494, MDA-MB231 ScriFL and T47D Scrib94-494 cells. **d)** Growth curves of cells ectopically expressing SCRIB FL and SCRIB 94-494, in the presence and absence of doxycycline. **e)** Cell cycle analysis by flow cytometry Cell cycle distribution of propidium iodide (PI)-labeled cells was analyzed by flow cytometric analyses after 72 h of SCRIB 94-494 induction by doxycycline 1 µg/ml. The peaks in the illustration correspond to the G1/G0, S and G2/M phases of the cell cycle. **f)** Measurement of plasma membrane phosphatidylserine externalization by flow cytometry using fluorescently labeled Annexin V FITC, for the detection of cell apoptosis **g)** Western blot analysis showing timecourse of Scrib 94-494 -tagged RFP ectopic expression in an inducible MDA-MB231 system, and downstream activation of caspase-3. Cells were induced by adding 1µg/ml doxycycline at time-points in growth medium.

4.2 The truncated Scrib activates JNK/c-Jun and p38-MAPK signaling axes.

It is well established that Scrib function as a specific regulator of several signal transduction pathways. To pinpoint the signals triggered by the expression of Scrib94-494 in MDA-MB231 cells 72h after induction, we used phospho-specific antibodies for key cellular signaling proteins. Several studies have shown that Scrib negatively regulates the phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) and positively regulates phosphorylation of c-Jun. Consistent with these reports, western blot analysis of whole-cell lysates of MDA-MB231 Scrib94-494 revealed that the truncated Scrib activated the c-Jun N-terminal kinase (JNK)/c-Jun and p38-MAPK signaling axes while it had no impact in MAPK-ERK1/2 signaling pathway, as indicated by phosphorylation status of these signaling proteins (Fig. 4.2b). Additionally, ectopic expression of Scrib94-494 abrogated the phosphorylation of the mitogen activated protein (MAP) kinase ERK5. To determine how universal is the capacity of Scrib94-494 to activate JNK/c-Jun and p38-MAPK signaling axes, we tested the effect of the

induction of Scrib94-494 in JNK pathway, in the breast cancer cell line T-47D as well as in a human glioblastoma cell line, called U-87MG (Fig. 4.2c). Western blot analyses of whole-cell lysates showed that phospho-c-jun and p38MAPK were both moderately activated, in response to Scrib94-494 expression in T-47D cells. Ectopic expression of the Scrib94-494, using a tet-on inducible system in U-87MG cells did not increase the phosphorylation status of c-Jun. Next, we examined if the overexpression of ScribFL has an effect in JNK pathway activation and we found that ectopic expression of the ScribFL resulted in a modest activation of the JNK pathway in MDA-MB231 cells (Fig. 4.2d).

ASK1, a MAPKKK that belongs to MAP kinase module has been shown to induce apoptosis via activation of JNK/c-Jun and p38 signaling pathways (Ichijo et al., 1997). Since, JNK and p38 signaling pathways were activated by the induction of Scrib94-494 expression, we tested whether selective inhibition of the ASK1 (Apoptosis Signal regulating Kinase) protein kinases of the MAP kinase module by the small-molecule inhibitors TC ASK10 impacted the Scrib94-494-mediated apoptosis. Cells were induced with doxycycline for 72h, either in the absence or presence of each inhibitor, and whole lysates were then analysed by western blot. Treatment of induced cells with the 20µM ASK1 inhibitor did not inactivate either JNK or p38-MAPK kinases and did not rescue the cells from Scrib94-494-mediated apoptosis (Fig. 4.2f). Whereas treatment of induced cells with 5µM SP600125 inhibitor inactivate the JNK/c-Jun signaling axis (Fig. 4.2e). These data indicate that the Scrib94-494-mediated activation signal for JNK and p38-MAPK is not induced by the ASK1 kinase, implying that another mechanism regulates these activations effects of the truncated Scrib.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ASK10
MAP3K
RAF
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MOS
TAK1
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MKK7
PAK1
MKK5
MAPK
ERK1
ERK2
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p38δ
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ERK3
ERK4
ERK5
MAPKAPK
MNK1
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SP600125
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MDA-MB231 Scrib94-494
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JNK
p-c-Jun Ser73
Tubulin
p-p38MAPK Thr180/Tyr182
p38MAPK
Tubulin
p-ERK5 Thr218/Tyr220
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p-ERK1/2 (p44/42)
ERK1/2 (p44/42)
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T-47D Scrib94-494
Dox - +
p-c-Jun Ser73
p-p38MAPK Thr180/Tyr182
p38MAPK
Tubulin
U-87MG Scrib94-494
Dox - +
p-c-Jun Ser73
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d
MDA-MB231 ScribFL
Dox - +
p-JNK1/2 Thr183/Tyr185
JNK
ScribFL
Tubulin
e
MDA-MB231 Scrib94-494
Inhibitors - - ASK10 SP600125
Dox - + + +
p-c-Jun Ser73
Tubulin
f
MDA-MB231 Scrib94-494
Inhibitors - - ASK10
Dox - + +
p-JNK1/2 Thr183/Tyr185
JNK
p-p38MAPK Thr180/Tyr182
p38MAPK
Cleaved caspase-3
Tubulin
g
Intracellular stimuli
Scrib94-494
p
p38
p
JNK

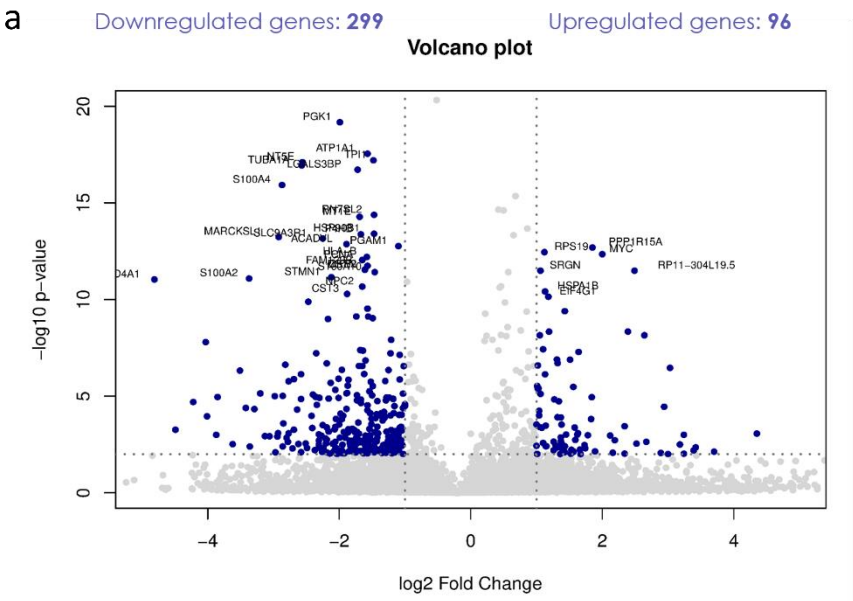
Figure 4.2. SCRIB 94-494 activates the JNK/c-Jun and p38-MAPK signaling axis in MDA-MB231 cells. Western blot analysis of whole-cell lysates of human cancer cell lines in which either Scrib94-494 or ScribFL expression was induced in the presence of doxycycline (+Dox). The activation of different signalling pathways was assessed using phospho-specific antibodies for activated kinases and their downstream targets. Any changes in total protein levels were also assessed. **a)** A schematic diagram of the MAP kinase module. Inhibitors of specific kinases within these signalling pathways are shown in red. **b)** Phosphorylation status of kinases of MAP kinases module in MDA-MB231 Scrib94-494 cells after doxycycline induction for 72h. When doxycycline is present: The JNK/c-Jun signaling axis is potently activated, whereas the MAPK p38 is moderately activated. ERK1/2 phosphorylation status is not affected and ERK5 phosphorylation is intensively reduced. **c)** In T47-D cell Scrib94-494 expressing cells moderate levels of JNK and p38 are detected. Whereas, in U-87MG JNK signaling pathway is not activated in response of Scrib94-494 expression. **d)** Overexpression of ScribFL in MDA-MB231 cells results in a moderate activation of the JNK signaling axis. **e)** Selective signalling inhibition of ASK1 and JNK kinases by TC ASK10 and SP600125 respectively. **f)** ASK1 inhibitor did not rescue cells from apoptosis. **g)** Working model suggesting that the truncated Scrib activates JNK and p38 in breast cancer.

4.3 Ectopic expression of Scrib 94-494 induces gene expression changes.

To better understand the cellular response to growth inhibition and apoptotic cell death-mediated, we used RNA-sequencing to investigate changes of the global transcriptional programme in MDA-MB231 Scrib94-494 cells upon treatment with doxycycline. Significant changes in gene expression occurred throughout the transcriptome (21.856 detected, 11.186 decreased and 10.670 increased). We found that cells ectopically expressing Scrib94-494 at levels approximately eighteen-fold greater than vector controls had a strong protein translation signature. A bioinformatics analysis of Scrib94-494-regulated signaling pathways and functions revealed enrichment of mTOR and endocytosis signaling as well as apoptotic cell death respectively. Furthermore, the analysis of Scrib94-494-regulated mRNAs revealed

elevated expression of RNAs encoding proteins that take part in protein synthesis, such as the EEF1G which constitutes a subunit of the elongation factor-1 complex, the EEF2 translation elongation factor, the initiation translation factor EIF4G1 and EIF3C, as well as several mitochondrial encoded tRNAs such as MT-TG, MT-TI, MT-TL1, MT-TV and the MRPS12 that constitutes a component of the mitochondrial small ribosomal subunit. These results indicate that the truncated SCRIB 94-494 controls a transcriptional program encoding components of the transcriptional machinery and several factors that regulate and promote mRNA translation.

a



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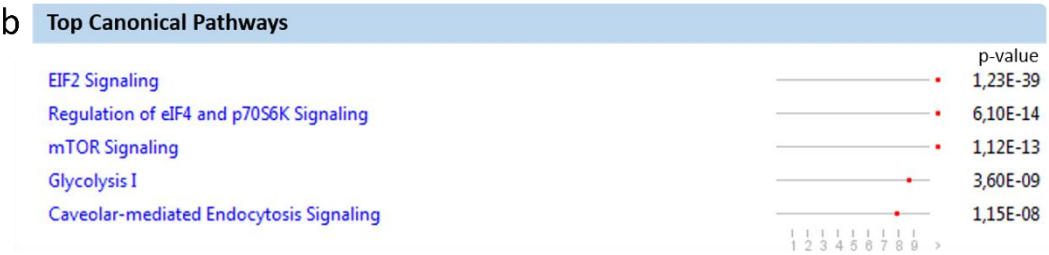


c

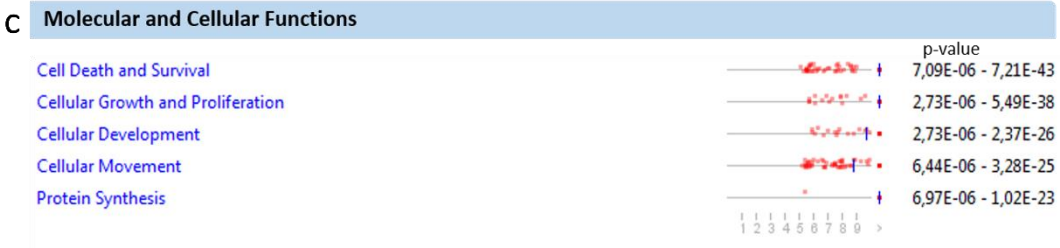


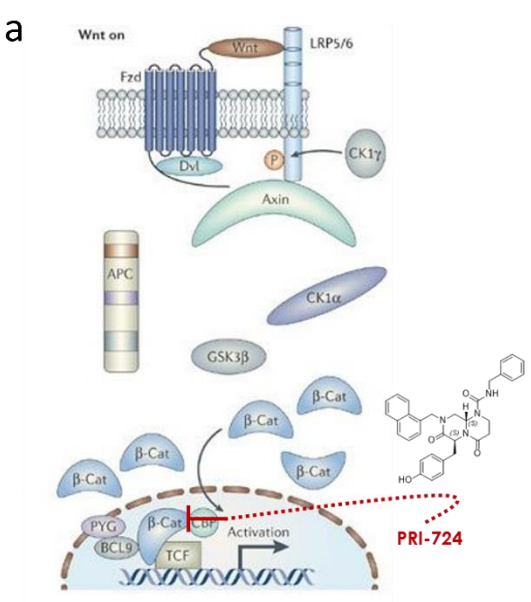
Figure 4.3. The truncated Scrib enhances the expression of RNAs that take part in the process of translation, including tRNAs, elongation factors and components of mitochondrial subunits. a) Global changes in mRNA levels following 72 h of doxycycline treatment in MDA-MB231 Scrib94-494 cells. Scrib94-494-mediated gene expression signature was analyzed by RNA-seq. **b)** Enriched canonical pathways, **c)** Enriched molecular and cellular functions, upon Scrib94-494 overexpression.

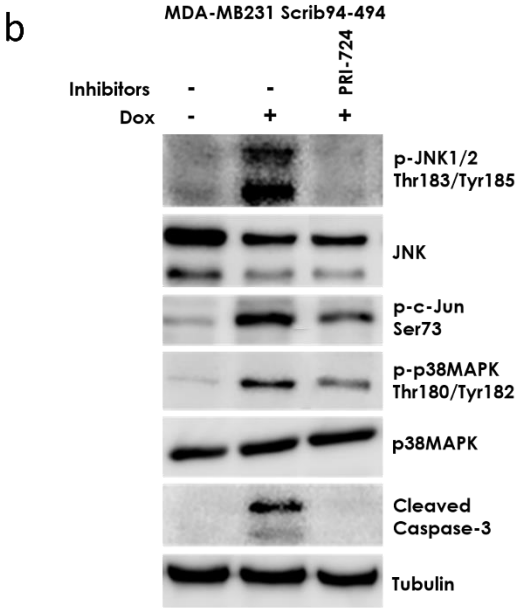
4.4 The truncated Scrib functions in Wnt signalling pathway.

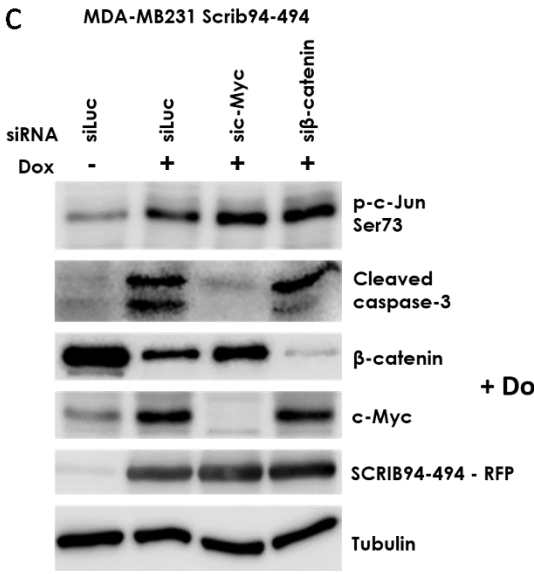
Several studies support the crosstalk among MAPK-mediated signaling and the canonical and non-canonical Wnt signaling (Bikavilli et al., 2009). It is well established that the canonical Wnt pathway involves activation of β-catenin-dependent transcription, promoting β-catenin stabilization through phosphorylation of GSK-3β at Ser9 (Lien et al., 2014). To test if JNK and p38-MAPK activation, mediated by the ectopic expression of Scrib94-494, necessitates a crosstalk among MAPK and Wnt/β-catenin signaling, we used a Wnt signaling pathway inhibitor, the PRI-724 that specifically inhibits the recruiting of β-catenin with its coactivator CBP, in MDA-MB231 Scrib94-494 cells. Treatment (72h) with the PRI-724 inhibitor (10μM) blocked the phosphorylation of JNK and p38-MAPK kinases and completely rescued the Scrib94-494-induced apoptosis (Fig. 4.4b). As we showed before, the ectopic expression of Scrib94-494 elevated gene expression of c-myc that constitutes a Wnt target. Interestingly, we observed that treatment with PRI-724 caused a reduction in protein levels of c-myc.

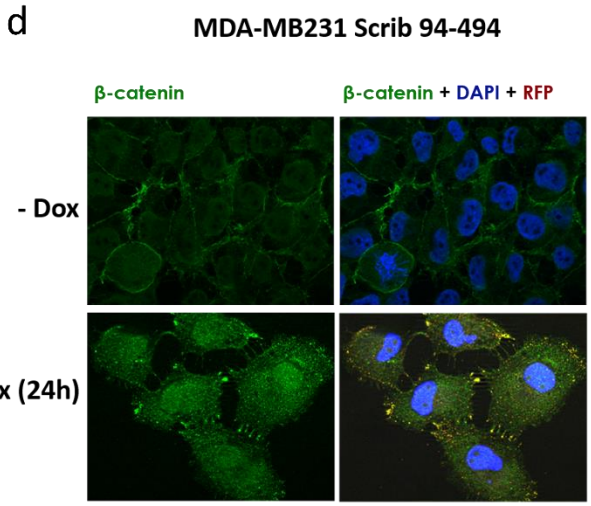
To further demonstrate the role of β-catenin activity in Scrib94-494-mediated apoptosis, we inhibited β-catenin and c-myc expression using a pool of three siRNAs for each one in MDA-MB231 Scrib 94-494 cells. Treatment of the cells with pooled β-catenin siRNAs for 72h showed that abrogation of β-catenin expression did not rescue the cells from Scrib94-494-induced cell death, whereas siRNA-mediated silencing of c-myc abrogates apoptosis in MDA-MB231 Scrib94-494 cells (Fig.4.4c). Consistent with our findings is that knockdown of α-catenin resulted in a dramatic reduction of anoikis of βPIX-knockdown in MDCK cells (Frank et al. 2012).

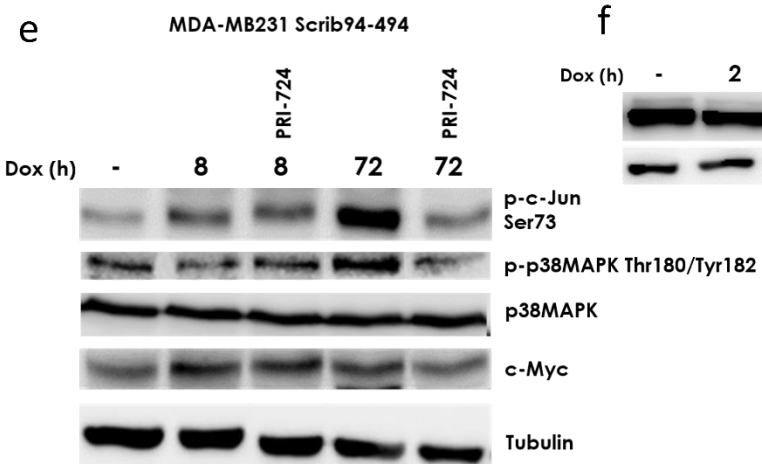
Immunofluorescence for endogenous β-catenin indicated its localization at cell-cell contacts in uninduced MDA-MB231 Scrib94-494 cells while in the presence of doxycycline (24h), β-catenin moves to the cytoplasm where it colocalizes with the truncated Scrib (Fig. 4.4d). Additionally, western blot analysis of whole-lysates showed a strong reduction of β-catenin protein levels after doxycycline induction for 24h in a time course experiment, presumably driven by the β-catenin destabilization. Next we showed that the reduction of β-catenin levels at 24h of induction comes along with strong phosphorylation of c-jun and p38-MAPK (Fig. 4.4e). Our data provide evidence for a β-catenin-mediated activation of a transcriptional program, emanating from Scrib94-494 that finally results in the activation of the JNK and p38MAPK kinases of the MAPK module. Collectively, our data imply a mechanism in which the Scrib94-494 initially necessitates β-catenin to exert its function but in a second phase the signaling events emanating from its expression results in β-catenin destabilization within 24h of induction with doxycycline.

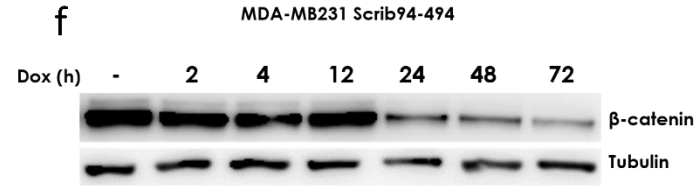

a
Wnt on
Wnt
LRP5/6
Fzd
Dvl
P
CK1γ
Axin
APC
CK1α
GSK3β
β-Cat
β-Cat
β-Cat
β-Cat
β-Cat
PYG
BCL9
TCF
Activation
PRI-724


b
MDA-MB231 Scrib94-494
Inhibitors - - PRI-724
Dox - + +
p-JNK1/2 Thr183/Tyr185
JNK
p-c-Jun Ser73
p-p38MAPK Thr180/Tyr182
p38MAPK
Cleaved Caspase-3
Tubulin


c
MDA-MB231 Scrib94-494
siRNA siLuc siLuc sic-Myc siβ-catenin
Dox - + + +
p-c-Jun Ser73
Cleaved caspase-3
β-catenin
c-Myc
SCRIB94-494 - RFP
Tubulin


d
MDA-MB231 Scrib 94-494
β-catenin
β-catenin + DAPI + RFP
- Dox
+ Dox (24h)


e
MDA-MB231 Scrib94-494
PRI-724
PRI-724
Dox (h) - 8 8 72 72
p-c-Jun Ser73
p-p38MAPK Thr180/Tyr182
p38MAPK
c-Myc
Tubulin


f
MDA-MB231 Scrib94-494
Dox (h) - 2 4 12 24 48 72
β-catenin
Tubulin

g

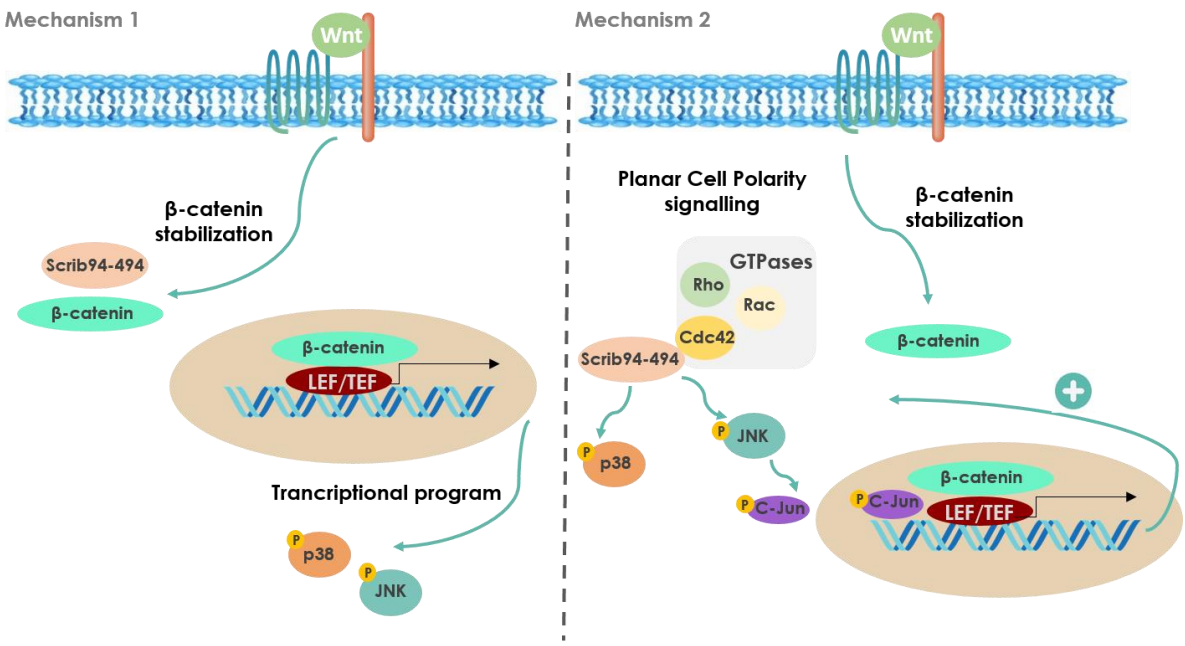


Figure 4.4. SCRIB 94-494 activates β-catenin-mediated transcriptional program in MDA-MB231 Scrib94-494 cells. Western blot analysis of whole-cell lysates of MDA-MB231 cells in which Scrib94-494 expression was induced in the presence of doxycycline (+Dox). The activation of JNK/c-jun and p38-MAPK signalling pathways was assessed using phospho-specific antibodies for activated kinases and their downstream targets. Any changes in total protein levels were also assessed. **a)** A schematic diagram of Wnt on signalling. Inhibitors within this pathway is shown in red. **b)** Phosphorylation status of kinases of MAP kinases module in MDA-MB231 Scrib94-494 cells after doxycycline induction for 72h. When doxycycline and PRI-724 are present: The JNK/c-Jun and MAPK signaling axes are inhibited. **c)** Western blot analysis of phosphor-c-jun Ser73, cleaved caspase-3, β-catenin and c-Myc in protein lysates of MDA-MB231 Scrib94-494 cells transfected with control or β-catenin or c-myc siRNA. **d)** Immunofluorescence detection of β-catenin in undinduced and induced for 24h with doxycycline MDA-MB231 Scrib94-494 cells. **e)** Western blot analysis for detection of phospho-p38MAPK and phospho-c-jun levels in a time-course experiment. **f)** Detection of β-catenin protein levels in a time-course experiment. **g)** Working model suggesting that the truncated Scrib activates JNK and p38 in a Wnt-dependent manner in breast cancer.

4.5 The truncated Scrib regulate β-catenin by promoting GSK3β phosphorylation.

Since, Glycogen synthase kinase 3β (GSK3β) constitutes an upstream regulator of β-catenin, we assessed the phosphorylation status of GSK3β. The N-terminus of GSK3β and GSK3a can be phosphorylated by several kinases, such Akt, p90RSK, protein kinase A (PKA), and p70S6 kinase, regulating their activity (Gao et al., 2014, Beurel et al., 2014). Additionally, it has been shown that p38MAPK is activated upon Wnt3a stimulation and is crucial for Wnt3a-induced accumulation of β-catenin through inhibiting GSK3β activity by inducing its phosphorylation at Ser9. GSK3β phosphorylation at Ser9 has been characterized as an inhibitory posttranslational modification of GSK3β, promoting β-catenin stabilization. A recent study, showed that Wnt3A induces not only the phosphorylation of GSK-3β at Ser9 and accumulation of β-catenin but also RhoA activation (Kim et al., 2016). In a time-course experiment, western blot analysis of whole-cell lysates indicated enhanced phosphorylation of GSK3β at its Ser-9, in MDA-MB231 Scrib94-494 cells induced for 8h with doxycycline, while strong decrease of phospho-GSK3β was observed in the induced for 72h cells. As we showed before, β-catenin protein levels drop at 24h of induction. These findings provide a mechanism by which β-catenin is regulated by the Scrib94-494-mediated phosphorylation and dephosphorylation of GSK3β at Ser9, in a time-dependent manner. We next sought to determine if the inhibition of CBP/β-catenin by the PRI-724 inhibitor affect the phosphorylation status of GSK3β. This analysis confirmed that phospho-GSK3β levels remained unchanged in the presence of the PRI-724 inhibitor, indicating that Scrib94-494-mediated regulation of GSK3β is modulated by a mechanism that function upstream of β-catenin/CBP transcriptional program. Furthermore, this finding reject the possibility that a feedback mechanism downstream of β-catenin/CBP transcriptional program could control GSK3β phosphorylation.

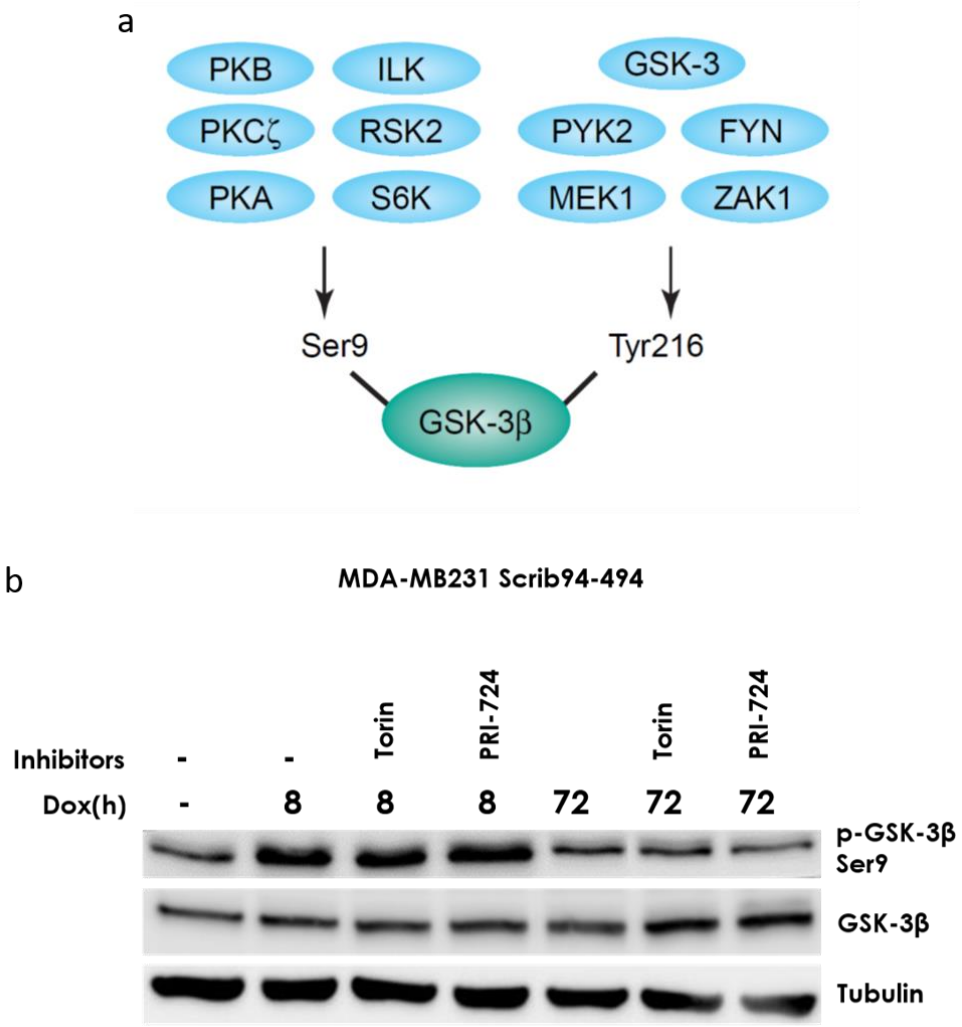


Figure 4.5. The truncated Scrib enhances GSK-3β phosphorylation at the site of Ser9. a) Schematic depiction of GSK-3β post-translational modifications. GSK-3b is regulated by post-translational phosphorylation of Ser9 and Tyr216. Phosphorylation of Ser9 can be carried out by p70S6K, p90rsk, protein kinase A (PKA), PKB (AKT), PKC isoforms and integrin-linked kinase (ILK). **b)** Western blot analysis of a time-course experiment of phospho-GSK-3 and total GSK-3β levels in MDA-MB231 Scrib94-494 induced and uninduced cells treated with Torin and PRI-724 inhibitors.

4.6 Scrib94-494 ectopic expression activates mTORC1 signaling pathway.

Our transcriptomic analysis results and a recent paper from another group (Feigen M. et al., 2014) hint that mutant Scrib might affect mTOR activity. To confirm these findings, we investigated the activation status of mTOR upon Scrib94-494 overexpression, assaying the phosphorylation status of direct mTORC1 substrates, such as S6K and 4E-BP1, using phospho-specific antibodies. Western blot analysis of whole-cell lysates showed elevated phospho-S6K levels, indicating elevated mTORC1 activity (Fig. 4b). Phosphorylation of S6K and 4E-BP1 mediates the transduction of mitogen and nutrient signals to stimulate translation. To determine how universal is the capacity of Scrib94-494 to activate mTOR signaling axis, we tested the effect of the induction of Scrib94-494 in mTOR pathway, in the breast cancer cell line T-47D (Fig. 2). Western blot analyses of whole-cell lysates showed that phospho-S6K were moderately elevated, in response to Scrib94-494 expression in T-47D cells compared to the MDA-MB231 cells. Next, we examined if the overexpression of ScribFL has an effect in mTOR pathway activation and we found that ectopic expression of the ScribFL resulted in a modest activation of mTORC1 pathway.

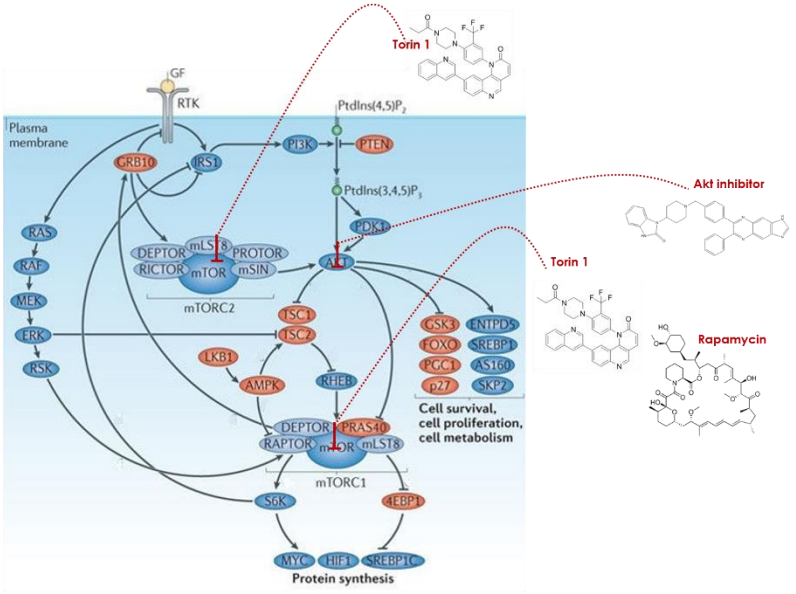
As described before, MDA-MB321 Scrib94-494 cells demonstrated apoptotic cell death and activation of JNK/c-Jun and p38-MAPK signaling axes, upon the expression of the truncated Scrib. To further examine the ability of Scri94-494 to activate mTORC1, as well as the involvement of mTOR activation in Scrib-mediated effects, we used the mTOR pharmacological inhibitors Rapamycin (20nM) and Torin 1 (250nM). Torin 1 is a potent and selective ATP-competitive inhibitor of mTOR (mammalian target of rapamycin) kinase, the catalytic subunit of the two functionally distinct complexes mTORC1 and mTORC2, that coordinately promote cell growth, proliferation, and survival. Torin 1 is able to effectively block phosphorylation mediated from both mTORC1 and mTORC2. Whereas, rapamycin acutely and directly inhibits mTORC1 and only chronic administration of rapamycin can inhibit mTORC2. Western blot analysis showed that, either in the presence of Torin 1 or Rapamycin, mTORC1 signaling was abrogated in MDA-MB231 Scrib94-494 cells treated with doxycycline for 72h, as indicated by a sharp decrease in phospho-S6K levels (Fig. 4c). Furthermore, Torin 1 but not Rapamycin strongly suppressed the Scrib94-494-

mediated activation of JNK/c-Jun and p38MAPK signaling axes, providing evidence that mTORC1 positively regulates JNK/c-Jun and p38MAPK. Also, we observed a moderate increase of phospho-Erk5 levels, in MDA-MB231 Scrib94-494 overexpressing cells when treated with Torin 1, something that did not happen when the cells were treated with Rapamycin (Fig. 4c). Additionally, we were not able to detect any caspase-3 cleavage upon mTOR inhibition, revealing a mechanism in a sequence in which the ectopic expression of the truncated Scrib results to the activation of apoptotic pathways through the activation of mTORC1.

We next tried to investigate, the upstream molecular mechanisms that regulate mTOR activation upon Scrib94-494 expression. To do so, we used specific phospho-antibodies to assess the phosphorylation status of Akt and TSC2, since Akt and TSC complex constitute upstream regulators of mTOR signaling pathway. Akt-mediated multi-site phosphorylation of TSC2 inhibits its ability to act as a GAP towards Rheb within cells, thereby allowing Rheb-GTP to activate mTORC1 signaling. PI3K regulates Akt activity by generating the lipid second messenger phosphatidylinositol-3,4,5-bisphosphate (PIP_3), which binds and recruits the Akt and PDK1 kinases to the plasma membrane. PDK1 phosphorylates the activation loop of Akt at Thr-308, which is essential for Akt activation. Western blot analysis of whole-cell lysates indicated a reduction of Akt phosphorylation at the of Thr-308 in MDA-MB231 Scrib94-494 cells induced with doxycycline for 72h, indicating reduced activity of Akt. Despite the activation of Akt by growth factor signals, mTOR kinase also function in Akt regulation. More specifically, when mTOR is associated with Rictor in the mTOR complex 2 (mTORC2), it directly phosphorylates Akt on Ser473, thereby contributing to Akt activation. So, for further testing of Akt activity we checked for the mTORC2-mediated phosphorylation Akt at Ser473 and we observed a moderate reduction of phosphor-Akt levels in the presence of doxycycline for 72h. To confirm our findings that the expression of the truncated Scrib does not result in Akt elevated activity, we checked the phosphorylation status of its downstream target TSC2 at the sites of Thr1462 and Se939. The phosphorylation of TSC2 at Thr1462 and Ser939 relieves the inhibitory effects of the TSC1-TSC2 complex on Rheb and mTOR complex 1 (mTORC1). Western blot analysis, of whole-cell lysates indicated no significant difference in phospho-TSC2 among the induced and the induced MDA-MB231 Scrib94-494 cells. Next we went

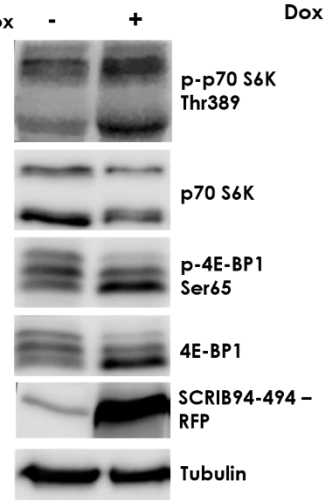
further and checked for phosphorylated sites of TSC2, mediated by other kinases. AMP-activated protein kinase (AMPK) is a major regulator of TSC1-TSC2 complex, exerting its action by phosphorylating the TSC2 at Ser1387, in response to low energy (high AMP/ATP ratio). We observed a moderate decrease of phospho-TSC2 Ser1387 in MDA-MB231 Scrib94-494 in the presence of doxycycline for 72h. Additionally, TSC2 was found to be phosphorylated on Ser1254 by MAPKAP kinase-2, which is activated downstream of p38MAPK (mitogen-activated protein kinase). Since, we have showed that p38MAPK is activated upon Scrib94-494 overexpression, we checked for phospho-TSC2 Ser1254 levels in MDA-MB231 Scrib94-494 induced cells and we observed a reduction of phosphor-TSC2 levels in the presence of doxycycline. Glycogen synthase kinase 3β (GSK-3β) activates the TSC complex by phosphorylating TSC2 at Ser1379 and Ser1383. Phosphorylation of these two residues requires priming by AMPK-dependent phosphorylation of Ser1387. Moreover, the involvement of the Akt kinase in Scrib94-494 mTOR activation was tested using the Akt inhibitor VIII, a chemical inhibitor against Akt. Pharmalogical Akt inhibition did not show to have any impact in the elevated activity of mTORC1 in MDA-MB231 cells in the presence of doxycycline. Our data, indicate that Scrib94-494-mediated activation of mTORC1 is Akt-independent and does not seem to be highly related to phosphorylation events of TSC2.

a



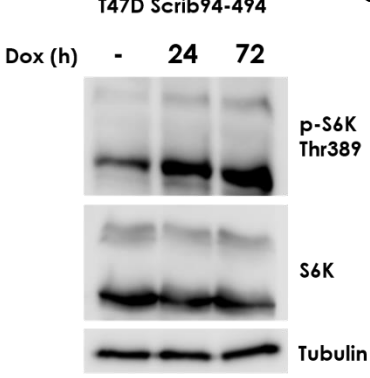
b

MDA-MB231 Scrib94-494



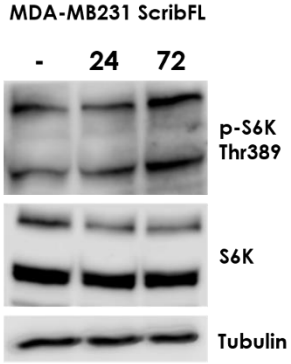
c

T47D Scrib94-494



d

MDA-MB231 ScribFL



d

MDA-MB231 Scrib94-494

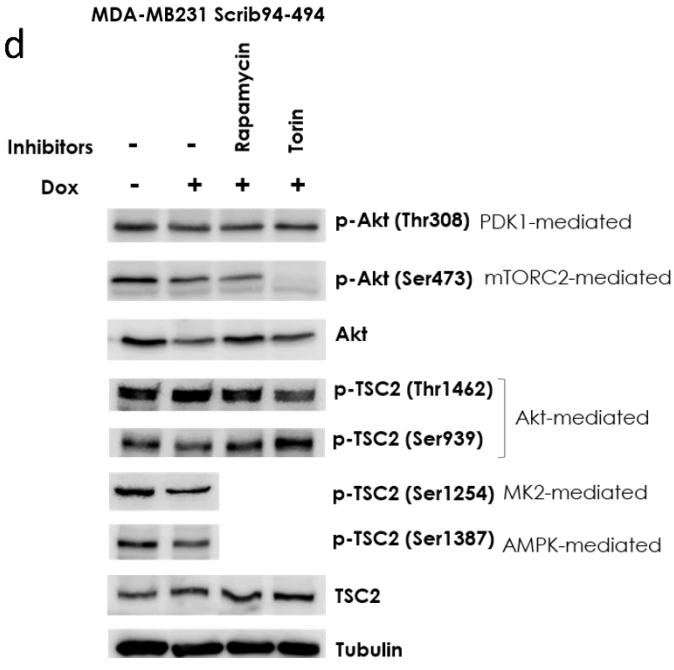


Figure 4.6.i Scrib 94-494 activates β-catenin-mediated transcriptional program in MDA-MB231 Scrib94-494 cells. Western blot analysis of whole-cell lysates of human breast cancer cell lines in which either Scrib94-494 or ScribFL expression was induced in the presence of doxycycline (+Dox). The activation of JNK/c-jun and p38-MAPK signalling pathways was assessed using phospho-specific antibodies for activated kinases and their downstream targets. Any changes in total protein levels were also assessed. **a)** A schematic diagram of mTOR signalling. Inhibitors within this pathway is shown in red. **b)** Phosphorylation status of mTORC1 targets after doxycycline induction for 72h in MDA-MB231 Scri94-494 cells. **c)** Phosphorylation status of mTORC1 targets in a time-course of doxycycline induction in T-47D Scrib94-494 cells. **d)** Phosphorylation status of mTORC1 targets in atime-course of doxycycline induction in MDA-MB231 ScribFL cells. **e)** Western blot analysis for detection of Akt and TSC2 phosphorylation status.

To pinpoint the crosstalk among Scrib94-494-induced signaling pathways, we used a panel of specific chemical inhibitors (Torin 1, Rapamycin, Akt, PRI-724) of the major signaling pathways activated by the truncated Scrib. Next, we sought to determine whether Scrib94-494-mediated cellular events and signaling pathways are affected by mTOR inhibition and whether blockage of the other Scrib94-494-induced affect mTORC1activity. We found that mTORC1activity is abrogated in the presence of Torin1 and Rapamycin inhibitors but is not affected by the inhibition of β-catenin/CBP transcription program, indicating that either mTORC1 functions upstream of β-catenin mediated signaling or that the truncated Scrib94-494 activate in parallel more than one signaling pathways. Interestingly, we found that the cells are rescued from apoptotic cell death when both by mTOR and β-catenin/CBP interaction inhibition, indicating that both mTOR and Wnt signaling are crucial for Scrib94-494-induced apoptosis. (Fig. 4.6.ii)

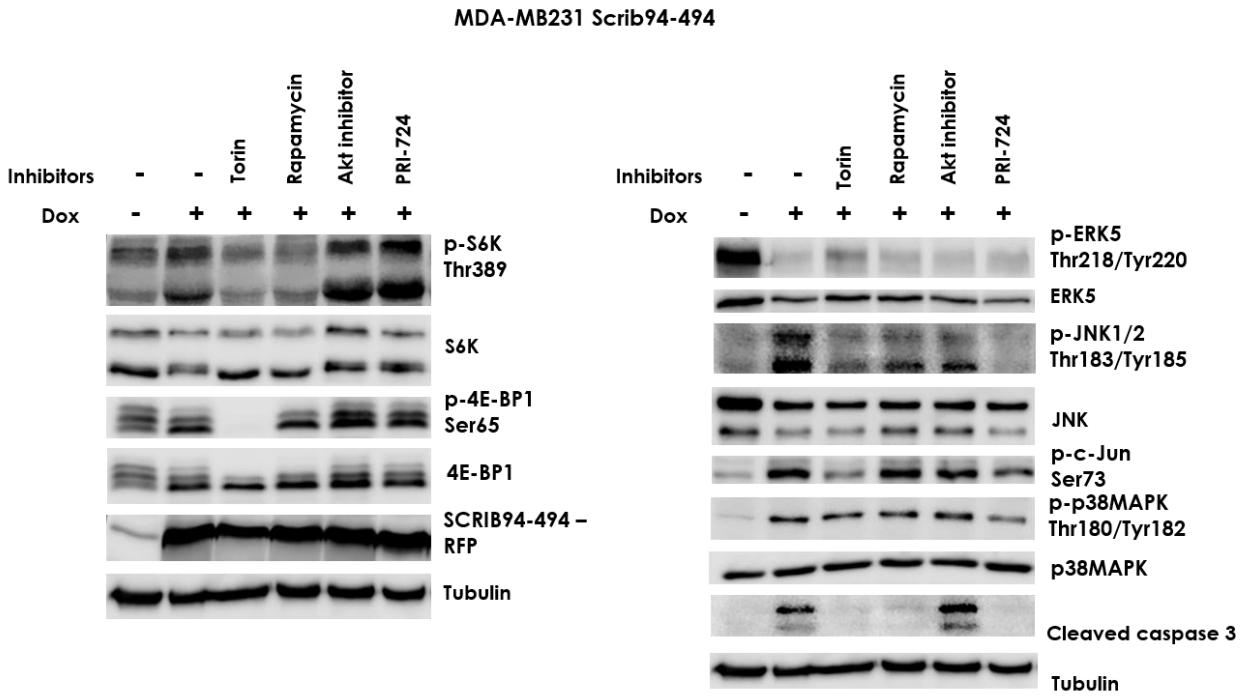


Figure 4.6.ii Scrib 94-494 mediated apoptosis necessitates both mTOR and Wnt signalling. MDA-MB231 Scrib94-494 induced cells were treated with a panel of specific chemical inhibitors for 72h.

4.7 c-Myc is essential for apoptotic cell death in MDA-MB231 Scrib94-494 cells.

Consistent with RNA-sequencing analysis and qRT-PCR which they revealed a 4-fold increase in c-Myc mRNA, western blot analyses of MDA-MB231 Scrib94-494 cells treated with doxycycline for 72h, showed elevated c-myc protein levels. A paper from another group, showed a Myc-induced increase in phospho-c-Jun protein levels in a Scribble-dependent manner demonstrating that Myc regulates the activation of Rac-JNK-Jun signaling pathway to induce apoptosis in MCF-10A cells (Zang et al. 2008). As we showed in our study, siRNA-mediated silencing of c-myc abrogates apoptosis in MDA-MB231 Scrib94-494 doxycycline induced cells, while enhances phospho-c-jun protein levels, indicating that c-Myc is essential for Scri94-494 induced apoptosis, possibly via a mechanism that does not require JNK signaling pathway activation. (Fig.4.7)

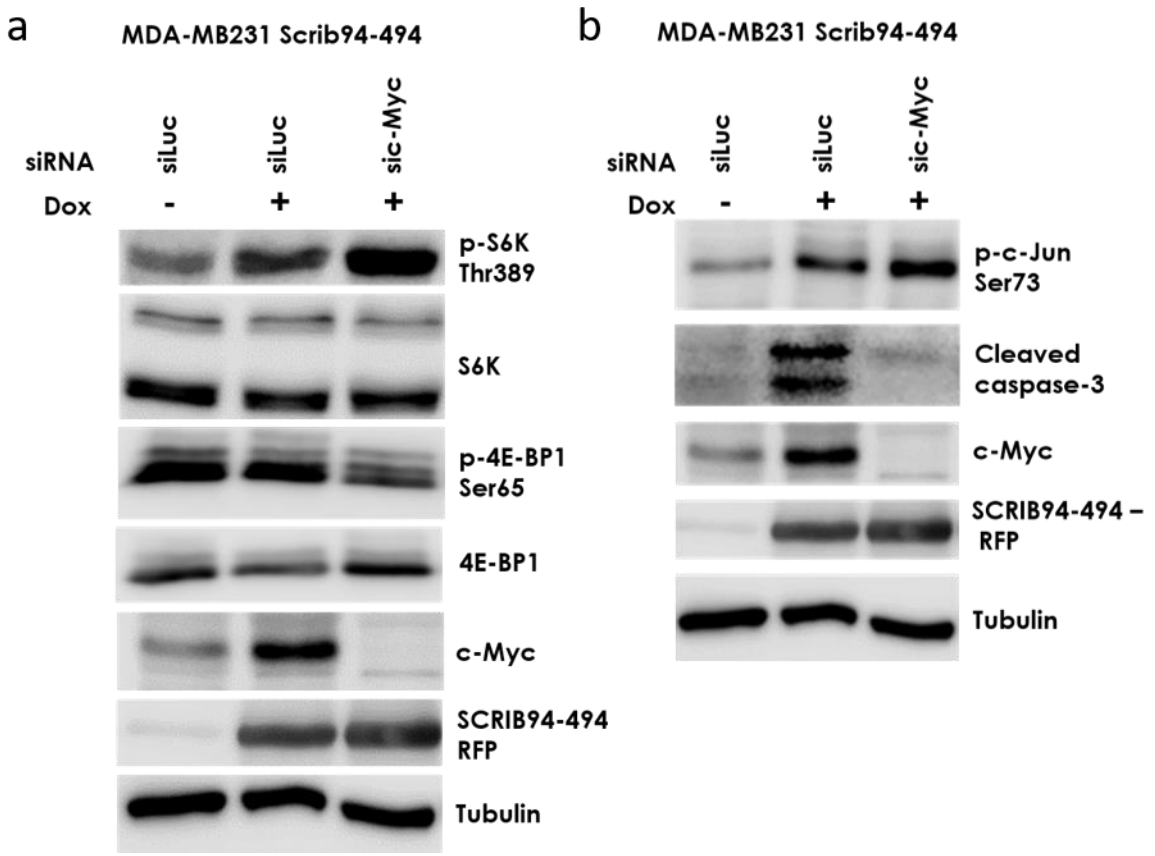
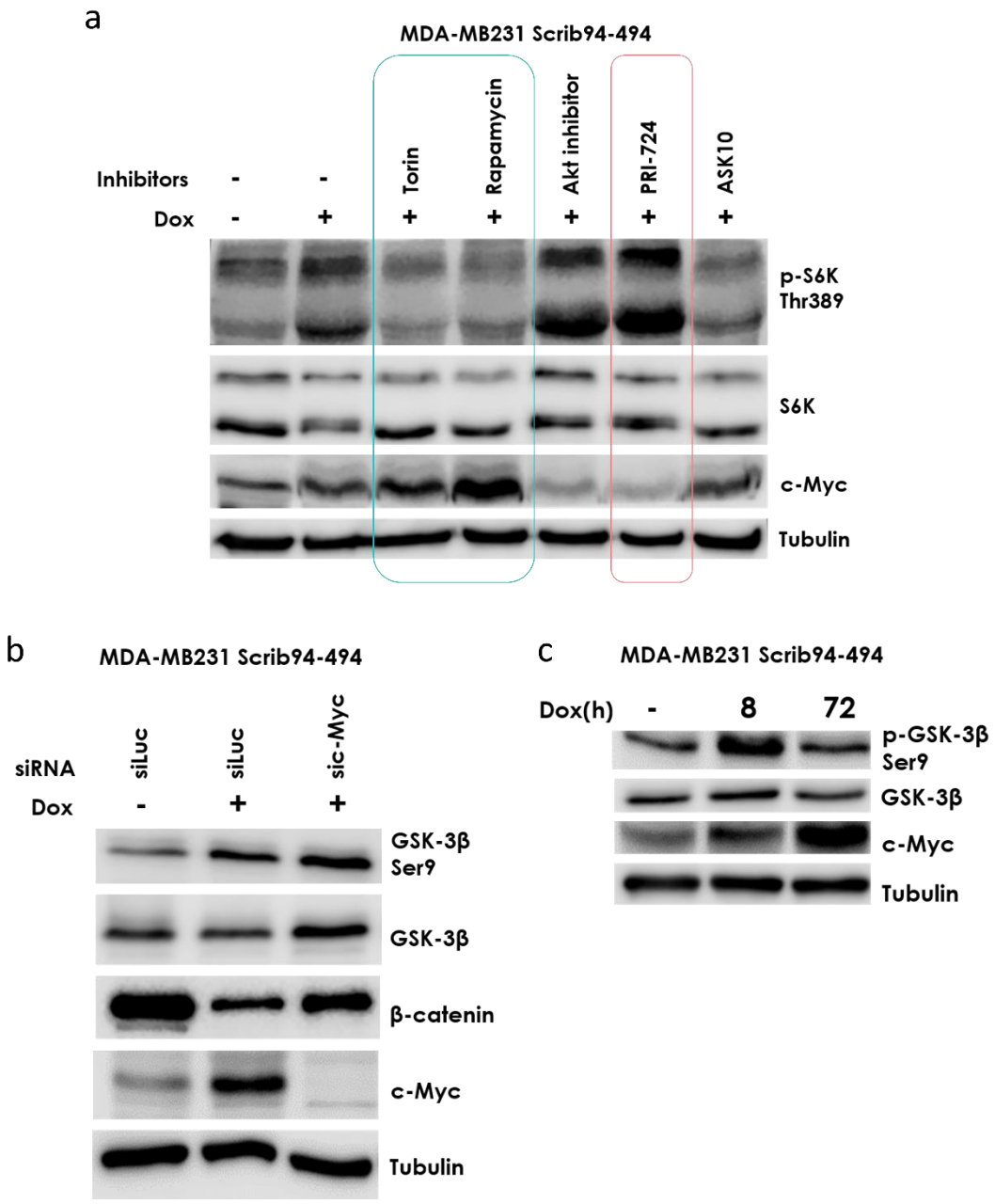


Figure 4.7. C-Myc depletion does not interfere in mTORC1 signaling, whereas abrogates apoptosis. Western blot analysis of whole-cell lysates of MDA-MB231 cells Scrib94-494, treated with doxycycline (+Dox) and siRNA against c-myc for 72h. The activation of different signalling pathways was assessed using phospho-specific antibodies for activated kinases and their downstream targets. Any changes in total protein levels were also assessed.

4.8 Wnt pathway is the key pathway in regulating c-myc levels.

Interestingly, as we showed before disruption of β-catenin/CBP binding using PRI-724 for 72h caused a reduction in protein and mRNA levels of c-myc in MDA-MB231 Scrib94-494 cells, treated with doxycycline. Treatment of induced cells with the Torin 1 inhibitor or Rapamycin did not reduced c-Myc protein levels. Our data suggest that Scrib94-494-induced c-Myc expression is dependent on β-catenin/CBP gene transcription and does not necessitate the activation of mTORC1signaling. (Fig. 4.8a)

Furthermore, it has been shown that induction of c-Myc in several cell types correlates with loss of the inhibitory serine 9 phosphorylation of GSK3β and its increased kinase activity, leading to β-catenin proteasomal degradation in a negative feedback mechanism (Liu et al., 2012). In a time-course experiment we showed that phospho-GSK-3β (Ser9) levels are significantly elevated at 8h of induction, while c-myc protein levels display a mild increase at the same time-point in MDA-MB231 Scrib94-494 induced cells. Interestingly, the induction for 72h revealed extremely reduced phospho-GSK-3β (Ser9) levels, whereas is showed to strongly upregulate c-myc protein levels (Fig.4.8c). To explore further if Scrib94-494-mediated c-myc expression negatively regulates the levels of phospho-GSK3β (Ser9) in cells depleted of c-myc by siRNA for 72h, we investigated the phosphorylation status of GSK-3β. Interestingly, we found that c-myc siRNA-mediated silencing did not alter the Scrib94-494-induced elevated phospho-GSK3β (Ser9) levels in MDA-MB231 Scrib94-494 induced cells, while strongly increased β-catenin protein levels, suggesting that in the absence of c-Myc, GSK3β remains inactive and unable to phosphorylate β-catenin that would lead to its proteasomal degradation (Fig.4.8b). Our data, suggest a mechanism of Scrib94-494 regulation of Wnt pathway, in a sequence in which the expression of the truncated Scrib increases the stability of β-catenin, resulting in elevated transcriptional activation by LEF-1/TCF transcription factors which in turns lead to elevated c-myc mRNA and protein levels that exert negative feedback actions on the phosphorylation of GSK-3β at the Ser9 site (Fig. 4.8 d).


a
MDA-MB231 Scrib94-494
Inhibitors - - + Torin + Rapamycin + Akt inhibitor + PRI-724 + ASK10
Dox - + + + + + +
p-S6K Thr389
S6K
c-Myc
Tubulin
b
MDA-MB231 Scrib94-494
siRNA siLuc siLuc sic-Myc
Dox - + +
GSK-3β Ser9
GSK-3β
β-catenin
c-Myc
Tubulin
c
MDA-MB231 Scrib94-494
Dox(h) - 8 72
p-GSK-3β Ser9
GSK-3β
c-Myc
Tubulin

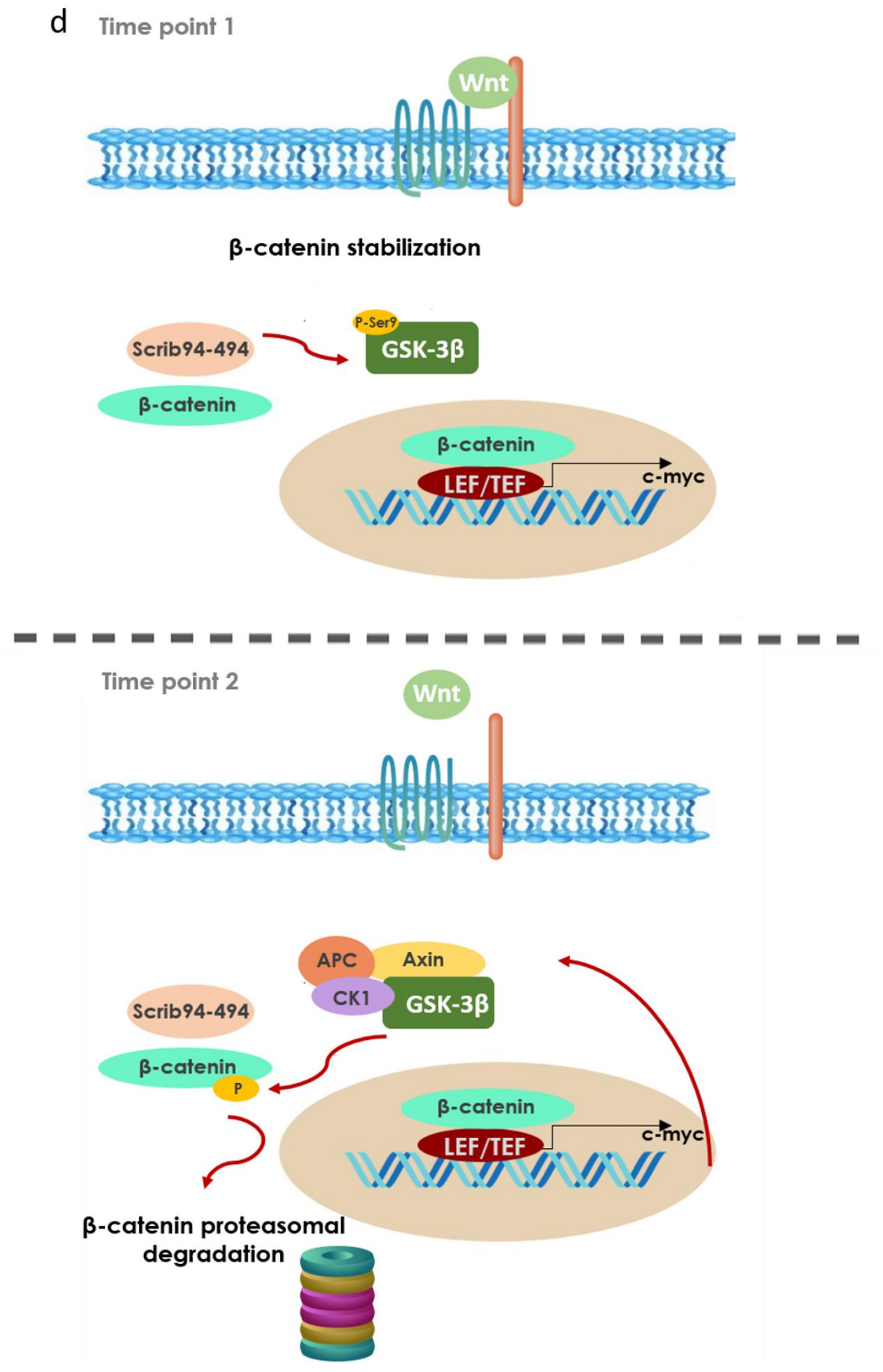

d
Time point 1
Wnt
β-catenin stabilization
Scrib94-494
P-Ser9
GSK-3β
β-catenin
β-catenin
LEF/TEF
c-myc
Time point 2
Wnt
APC
Axin
CK1
GSK-3β
Scrib94-494
β-catenin
P
β-catenin
LEF/TEF
c-myc
β-catenin proteasomal degradation

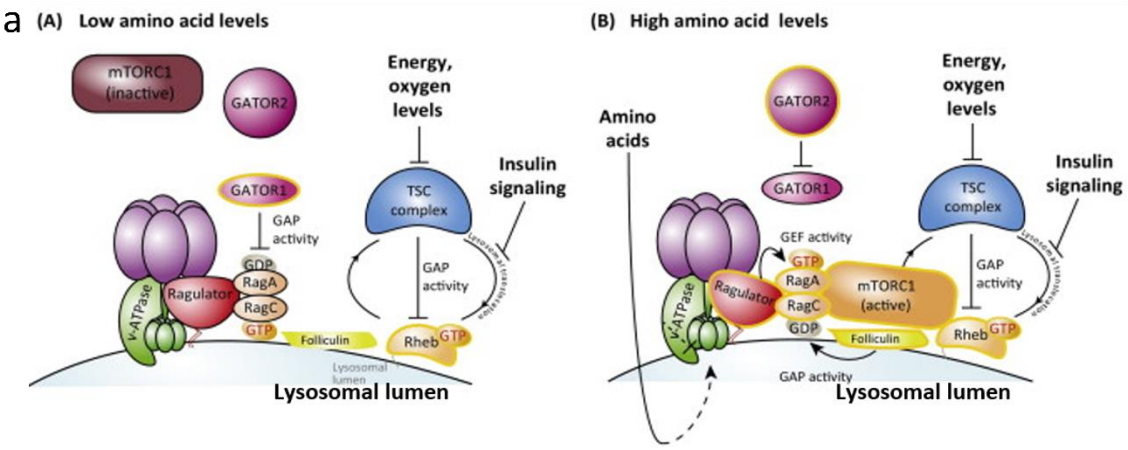
Figure 4.8. C-Myc expression is dependent on β-catenin/CBP gene transcription. **a)** Western blot analysis of whole-cell lysates of MDA-MB231 cells Scrib94-494, treated with doxycycline (+Dox) and Torin, Rapamycin, Akt, PRI-724, ASK10 inhibitors for 72h. The activation of mTORC1 signaling pathway was assessed using phospho-specific antibodies for the S6K kinase. Any changes in total protein levels of total S6K and c-Myc protein levels were also assessed. **b)** Western blot analysis of whole-cell lysates of MDA-MB231 cells Scrib94-494, treated with doxycycline (+Dox) and siRNA against c-myc for 72h. **c)** Time-course of phospho-GSK3β, GSK3β total and c-Myc protein levels. **d)** Proposed mechanistic model.

4.9 Scrib94-494-mediated activation of mTORC1 does not respond to amino-acid starvation.

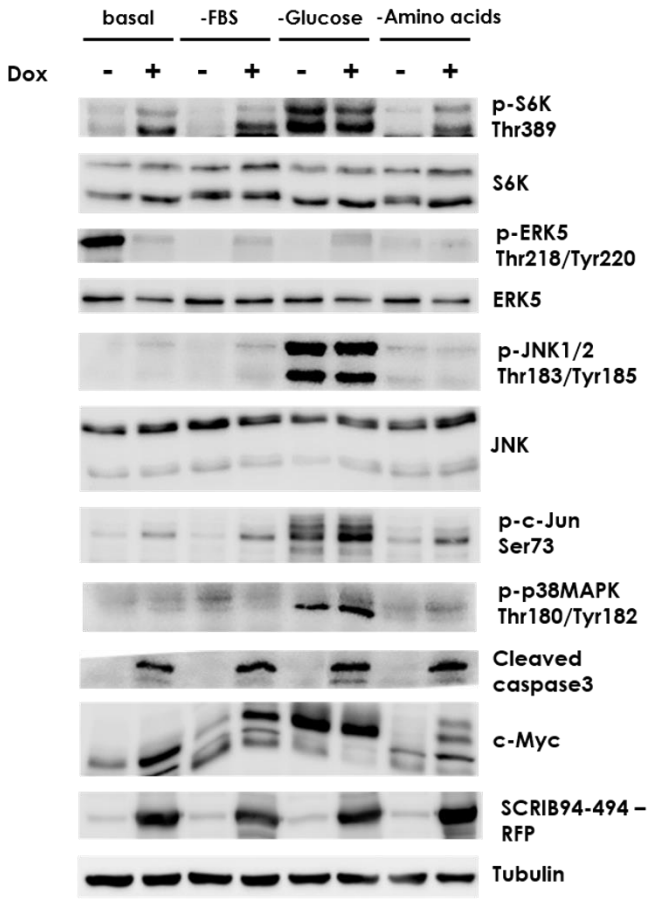
mTORC1 integrates signals from growth factors, nutrient and ATP availability to control protein, lipid and nucleic acid synthesis. Building blocks of macromolecules, such as amino acids and nucleotides activates mTORC1 pathway. Amino acids stimulate mTORC1 by promoting the conversion of RAS-related GTP-binding protein (RAG) heterodimers to the active con- formation, in which RAGA or RAGB is loaded with GTP and RAGC or RAGD is loaded with GDP. The active RAG heterodimer recruits mTORC1 to the surface of the lysosome where mTORC1 binds RHEB. To determine whether the activation effect of the truncated Scrib is regulated by growth factor or nutrient signaling, we tested the phosphorylation status of downstream targets of mTOR under starvation cell culture conditions, in the absence of serum, amino-acids or glucose from the growth medium. Interestingly, we found that the truncated Scrib is able to activate mTORC1 independently of growth factor and amino-acid availability, as indicated by increased phospho-S6K levels in both conditions. Next, we found that either serum or amino-acid starvation does not abrogate the impact of Scrib94-494 in MAPK kinases module. To our surprise, glucose deprivation seems to mimic in an extent the Scrib94-494-mediated molecular events.

To investigate if Scrib94-494-mediated mTORC1 activation is independent of amino-acid sensors, we carried out siRNA silencing of the Rag GTPAases in MDA-MB231 Scrib94-494 cells. We found that either RagA+B or RagC+D siRNA-mediated silencing did not impact in Scrib94-494-mediated activation of mTORC1 and MAPK kinase module and did not rescue the cells from apoptosis (Fig.4.9b). Next, to further examine the capacity of the truncated Scrib to modulate mTORC1 activity, we carried out siRNA-mediated silencing of the Rheb GTPase. Rheb depletion revealed an impaired Scrib94-494-mediated modulation of MAPK module, indicating that mTOR activation is crucial for the enhancement of Scrib94-494-mediated effect on MAPK module. Furthermore, siRNA-mediated Rheb depletion followed by a reduction of c-myc protein levels, suggesting that its expression is under the control of mTORC1 pathway. Interestingly, we showed that Rheb depletion rescues induced MDA-MB231 Scrib94-494 cells from apoptosis, confirming our previous findings that Scrib94-494-induced apoptotic cell death necessitates mTORC1 activation (Fig.4.9c).

a


(A) Low amino acid levels
mTORC1 (inactive)
GATOR2
GATOR1
GAP activity
GDP
RagA
RagC
GTP
Ragulator
V-ATPase
Folliculin
Rheb GTP
Energy, oxygen levels
TSC complex
GAP activity
Insulin signaling
Lysosomal lumen
(B) High amino acid levels
Amino acids
GATOR2
GATOR1
GEF activity
GTP
RagA
RagC
GDP
mTORC1 (active)
Ragulator
V-ATPase
Folliculin
Rheb GTP
GAP activity
Energy, oxygen levels
TSC complex
GAP activity
Insulin signaling
Lysosomal lumen

b


MDA-MB231 Scrib94-494
basal
-FBS
-Glucose
-Amino acids
Dox
- + - + - + - +
p-S6K Thr389
S6K
p-ERK5 Thr218/Tyr220
ERK5
p-JNK1/2 Thr183/Tyr185
JNK
p-c-Jun Ser73
p-p38MAPK Thr180/Tyr182
Cleaved caspase3
c-Myc
SCRIB94-494 – RFP
Tubulin

c

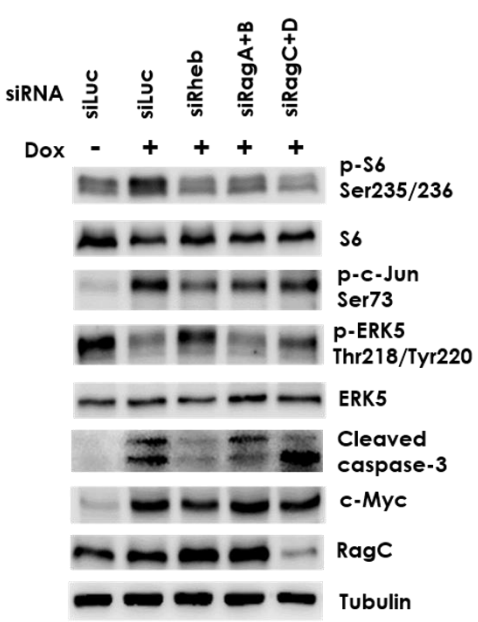
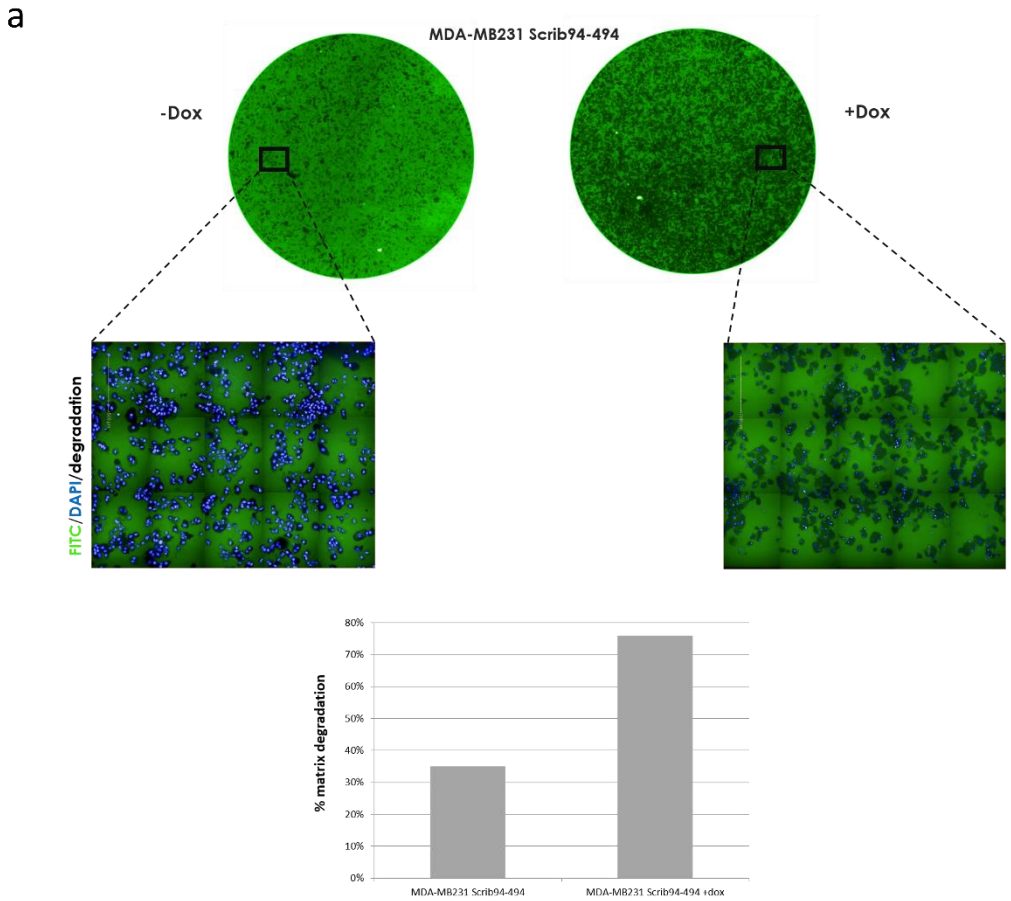

MDA-MB231 Scrib94-494
siRNA
siLuc
siLuc
siRheb
siRagA+B
siRagC+D
Dox
- + + + +
p-S6 Ser235/236
S6
p-c-Jun Ser73
p-ERK5 Thr218/Tyr220
ERK5
Cleaved caspase-3
c-Myc
RagC
Tubulin

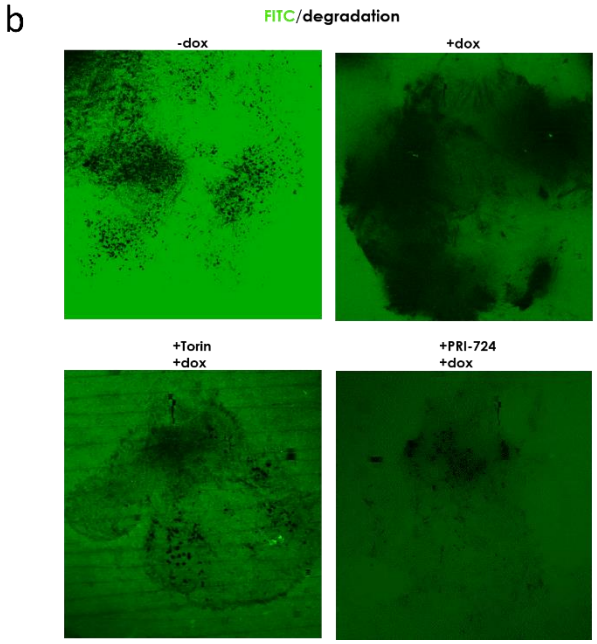
Figure 4.9. The truncated Scrib activates mTORC1 independently of growth factor stimulus and the action of Rag GTPases. a) Schematic depiction of mTORC1 activation on lysosome surface. **b)** Western blot analysis of Scrib94-494-mediated effects, upon FBS, Glucose and Amino acid starvation. **c)** Western blot analysis of Scrib94-494-mediated effects, upon siRNA depletion of Rheb and Rags GTPases.

4.10 Ectopic overexpression of the truncated Scrib enhances matrix degradation of ECM of MDA-MB231 cells but inhibits their stem cell properties.

Dow et al. showed that loss of Scrib promotes cell invasion through the activation of the MEK1/2-ERK1/2 signaling axis (Dow et al., 2008), implying a role of Scrib in invasiveness properties of tumor cells. Given the link between Scrib loss and cell invasion, we decided to test if the ectopic expression of Scrib94-494 impact on invasiveness properties of MDA-MB231 cells. To address this question, MDA-MB231 cells were seeded on gelatin-FITC-coated coverslips without or in the presence of doxycycline and matrix degradation quantified by fluorescent microscopy. Induced MDA-MB231 Scrib94-494 displayed elevated capacity to degrade the gelatin-FITC matrix and alterations in the pattern of degradation compared to untreated cells. It is proven that a variety of cancer cell lines degrade matrix at membrane protrusions such as invadopodia (dot-shaped degradation), as well as at numerous peripheral sites that represent bona fide Focal Adhesion sites (stick-like degradation) (see paragraph 1.1.3.1). We showed that uninduced cells degrade the fluorescent matrix in a dot-shaped and stick-shaped manner, whereas induced cells display a diffuse pattern of matrix degradation similar with podosome rosette–associated ECM degradation (Fig. 4.10a) (Pan et al. 2011). Torin and PRI-724 inhibitors were capable of significantly blocking the Scrib94-494-induced matrix degradation (Fig. 4.10b). To clarify if Scrib94-494-mediated alterations in matrix degradation are associated with changes in membrane protrusions, we carried out transmission electron microscopy which revealed cell shrinkage, reaching agreement with the apoptotic phenotype that MDA-MB231 cells acquire upon Scrib94-494 overexpression (Fig 4.10c). Using a mammosphere formation assay where we cultured the cells under non-attachment

conditions, we tested if the ectopic expression of Scrib94-494 affects cancer stem cell properties of MDA-MB231 cells. We showed that the formation of mammospheres was abolished in the presence of doxycycline, in MDA-MB 231 cells (Fig 4.10d).





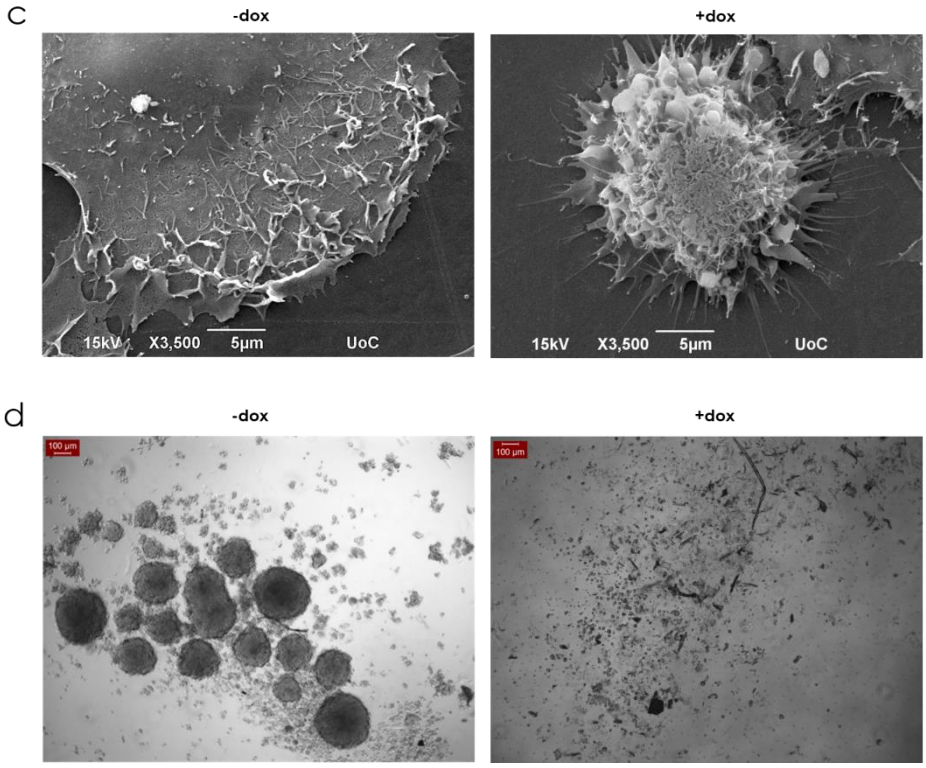


Figure 4.10. The truncated Scrib enhances the proteolytic activity of MDA-MB231 cells. a) MDA-MB231 Scrib94-494 cells were seeded on FITC-coupled gelatin-coated coverslips. After 72 hours of induction, cells were fixed and microscopically examined. Quantification of FITC-coupled gelatin degradation by MDA-MB231 Scrib94-494 cells in the presence or absence of doxycycline. **b)** MDA-MB231 Scrib94-494 were seeded on FITC-coupled gelatin-coated coverslips in the presence of doxycycline and the inhibitors Torin and PRI-724. **c)** Transmission electron microscopy of induced and induced MDA-MB231 Scrib94-494 cells. **d)** Mammosphere formation assay of MDA-MB231 Scrib94-494 cells in the presence or absence of doxycycline. MDA-MB231 Scrib94-494 overexpressing cells does not display proliferative ability in non-adherent culture conditions. In contrast, MDA-MB231 cells formed numerous colonies.

4.11 The truncated Scrib regulates protein levels of Notch receptors.

A number of research findings make the polarity determinant Scrib and the fate determinant Numb crucial regulators of the balance between membrane receptor recycling and trafficking toward lysosomes (see paragraph 1.1.8). Furthermore, it has

been shown that there is a link between mutant Scrib and oncogenic Notch in tumor growth in *Drosophila* (Brumby et al. 2003). To examine the effects of ectopic expression of Scrib94-494 on Notch signaling, we used Notch isoform-specific antibodies that detect both the full-length and the NTM region which consists of a short extracellular juxtamembrane peptide, a transmembrane sequence and the intracellular domain (NICD). In a time-course experiment we showed that Notch1 protein levels are significantly reduced at 48h of induction in MDA-MB231 Scrib94-494 induced cells (Fig. 4.11a). Next, to examine if the protein levels of other Notch isoforms are affected after 72h of induction, we used isoform-specific antibodies for Notch2 and Notch3 and we observed a similar reduction of their protein levels (Fig. 4.11b). We next tested whether, the Scrib94-494-mediated activation of mTOR and Wnt signaling pathways play a role in the regulation of Notch protein levels. To do so, we checked Notch protein levels in induced MDA-MB231 Scrib94-494 cells in the presence of a panel of pharmacological inhibitors. We showed that in the presence of Torin, Notch1 protein levels were increased, whereas in the presence of PRI-724, Notch1 protein levels remained reduced (Fig. 4.11c). Furthermore, siRNA-mediated silencing of Rheb increases Notch1 protein levels in MDA-MB231 Scrib94-494 doxycycline induced cells, reinforcing the involvement of mTOR signaling pathway in the regulation of Notch protein levels (Fig. 4.11d). To test if other cell polarity determinants have an effect on the regulation of Notch signaling, we decided to use MDA-MB231 cells that overexpress stably the cell fate determinant Numb4, as well as MDA-MB231 cells that overexpress stably the *Drosophila* gene PON (aa450-623) that constitute the adaptor of Numb in *Drosophila*. We showed that ectopic overexpression of either Numb4 or PON (aa450-623) results in significant decrease of Notch isoforms protein levels (Fig. 4.11e). Such reduction of Notch protein levels could imply a general mechanism of action of cell polarity determinants in the regulation of cell surface receptors levels by controlling their trafficking. To elucidate if *Drosophila* PON (aa450-623) interact with endogenous Numb in mammalian system, HEK 293T were transiently transfected with PON (aa450-623). Immunoprecipitation-western blot analysis revealed that *Drosophila* PON (aa450-623) interacts with endogenous Numb in mammalian cells (Fig. 4.11f).

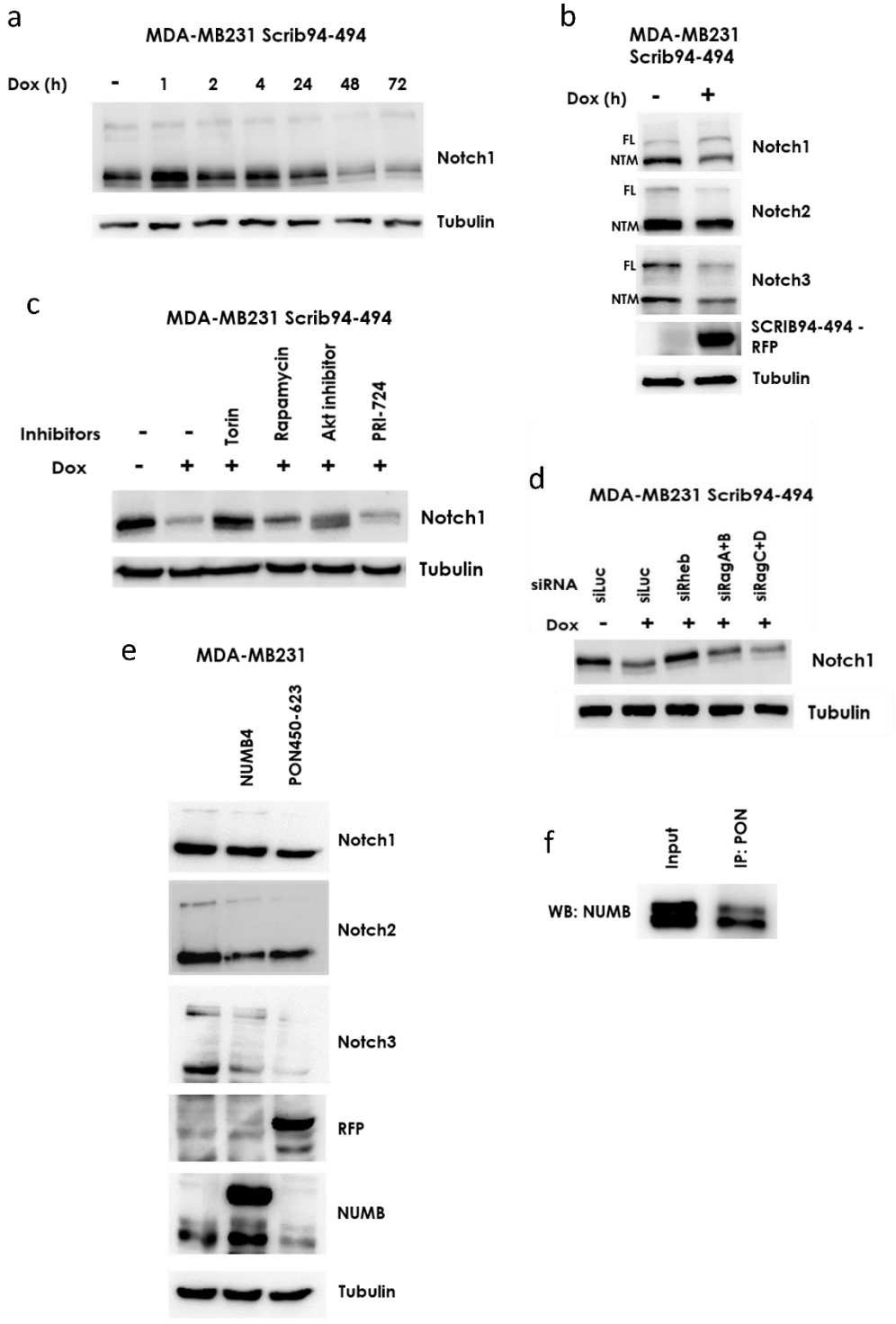

a
MDA-MB231 Scrib94-494
Dox (h) - 1 2 4 24 48 72
Notch1
Tubulin
b
MDA-MB231
Scrib94-494
Dox (h) - +
FL
NTM
Notch1
Notch2
Notch3
SCRIB94-494 - RFP
Tubulin
c
MDA-MB231 Scrib94-494
Inhibitors - - Torin Rapamycin Akt inhibitor PRI-724
Dox - + + + + +
Notch1
Tubulin
d
MDA-MB231 Scrib94-494
siRNA siLuc siLuc siRheb siRagA+B siRagC+D
Dox - + + + +
Notch1
Tubulin
e
MDA-MB231
NUMB4
PON450-623
Notch1
Notch2
Notch3
RFP
NUMB
Tubulin
f
Input
IP: PON
WB: NUMB

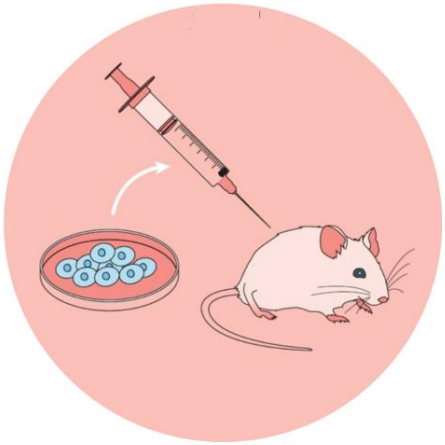
Figure 4.11. SCRIB 94-494 reduces Notch1 protein levels through mTORC1 activation. Western blot analysis of whole-cell lysates of MDA-MB231 stable cell lines that ectopically express cell polarity determinants. The protein levels of Notch isoforms were assessed using isoform-specific antibodies for Notch1, Notch2 and Notch3 receptors. **a)** Western blot analysis showing Notch1 protein levels in a timecourse experiment in MDA-MB231 Scrib94-494 cells. **b)** Notch isoform protein levels in MDA-MB231 Scrib94-494 cells after doxycycline induction for 72h. When doxycycline is present, Notch protein levels are intensively reduced. **c)** MDA-MB231 Scrib94-494 induced cells were treated with a panel of specific chemical inhibitors for 72h. When Torin is present Notch protein levels are increased. **d)** Western blot analysis of Scrib94-494-mediated alterations of Notch1 protein levels, upon siRNA depletion of Rheb and Rags GTPases **e)** Western blot analysis showing Notch1 protein levels in MDA-MB231 Numb4 GFP-tagged and MDA-MB231 PON (aa 450-623) RFP-tagged stable cell lines. **f)** In HEK 293T cells, *Drosophila* PON (aa 450-623) interacts with endogenous Numb.

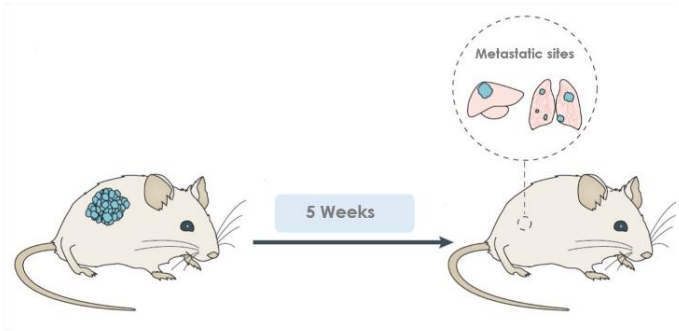
4.12 Scrib protein in breast cancer and metastasis

According to TCGA publically available genomic data, SCRIB is amplified in many invasive and highly aggressive tumors, such as breast and lung adenocarcinoma (Lin et al. 2015). To get insight into the invasive behavior of a human breast cancer cell line in which SCRIB gene is amplified, we used MDA-MB231 cells in an ectopic xenograft model. To do so, MDA-MB231 breast cancer cells stably expressing GFP were injected subcutaneously into immunocompromised mice and after approximately 5 weeks, mice were sacrificed and tested for organ specific metastases. Metastatic tumor sites were observed by optical imaging, in isolated lung and liver tissues and in the peripheral blood as well (Fig. 4.12b). Primary tumor cells, colonized tumor cells at sites of metastasis as well as groups of circulating tumor cells detected in blood were isolated and then cultured under 2D conditions. Next, using real-time PCR we found that cells isolated from different metastatic sites display significantly different

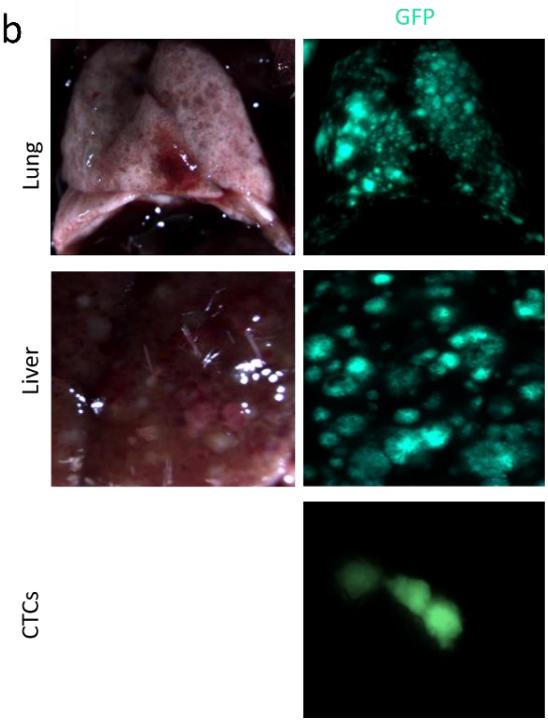
expression levels of several genes that display fluctuations of expression during EMT. Among the preferentially expressed cell adhesion molecules, we found E-cadherin, EpCAM and CD24. Tumor cells, in order to invade either undergo Epithelial-to-Mesenchymal Transition (EMT) characterized by loss of cell-cell adhesion or they display collective migration with maintained cell-cell contacts. It is widely known that Epithelial-to-Mesenchymal Transition (EMT) is driven by the action of the transcription factors SNAIL, ZEB and TWIST that repress the expression of the cell-cell adhesion molecules, such as E-cadherin while activate the expression of genes associated with the mesenchymal phenotype, such as Vimentin and N-cadhrerin (Lamouille et al., 2014). Additionally, CD24 has been shown to induce EMT in ovarian cancer (Nakamura et al., 2017) and a correlation between expression of EMT-associated markers and CD24 has been reported in pancreatic cancer (Zhang et al. 2012). It is also proven that Scrib overexpression results in reduced expression levels of the EMT inducers, promoting the expression of tight junctions (Elsum et al., 2013). A hypothetical model is that amplified Scrib in MDA-MB231 cells strengthens cell-cell adhesion, promoting collective migration without losing cell-cell contact. Our results can suggest that Scrib amplification may have a role in collective migratory response of MDA-MB231 cells. Furthermore, our results imply that MDA-MB231 tumor cells undergo gene expression changes which underlines the tumor cell plasticity during the process of metastasis, as well as the molecular adaption of tumor cells on the tissue microenvironment. Moreover, it could be the result of the heterogeneity of cancer cells that make up a tumor mass.

a




Metastatic sites
5 Weeks

b


GFP
Lung
Liver
CTCs

c

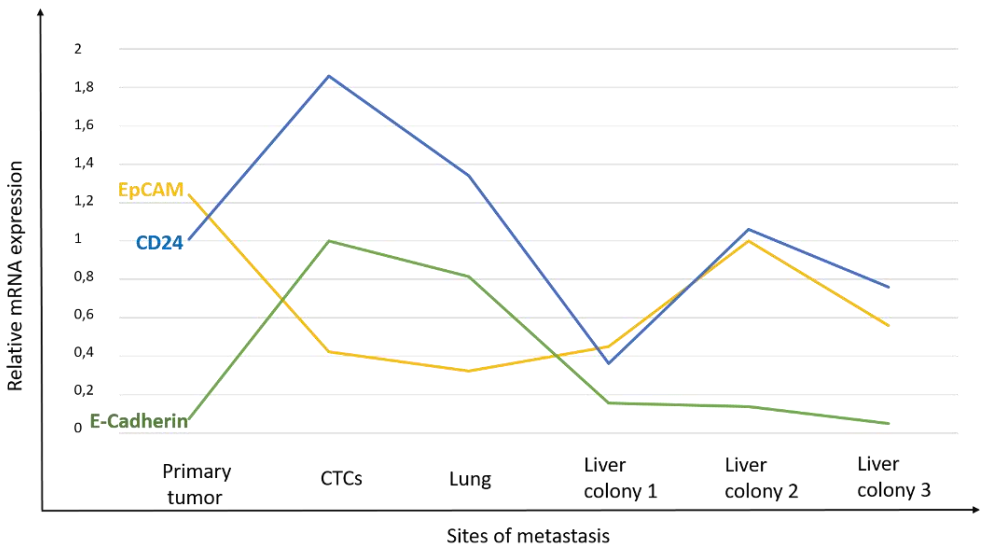

Relative mRNA expression
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
EpCAM
CD24
E-Cadherin
Primary tumor
CTCs
Lung
Liver colony 1
Liver colony 2
Liver colony 3
Sites of metastasis

Figure 4.12. Tumor cells undergo gene expression changes during the process of metastasis. a) Schematic representation of tumor xenograft experiment. **b)** Metastatic tumors as visualized by a stereoscope under brightfield and fluorescent conditions, as well as CTCs from peripheral blood as visualized by a fluorescence microscope. **c)** EpCAM, CD24 and E-cadherin relative mRNA levels among tumor cells isolated from primary tumor, peripheral blood as well as metastatic sites and cultivated in 2D cultures.

5. Discussion

Several studies have shown that perturbations in cell polarity can lead to hyperplasia but are insufficient to drive tumor formation alone and the subscription of activated oncogenic signaling pathways promotes the emergence of the malignant phenotype of cells (Zhan et al., 2008, Feigin et al., 2014). In human cancers, SCRIB gene has been found to be mutated, overexpressed or amplified and Scrib protein is commonly mislocalized in histopathological tumor samples. Mislocalization of Scrib phenocopies loss of Scrib expression and leads to perturbations of cell polarity, adhesion junctions disruption as well as enhancement of growth and proliferation signaling. We observed that a truncated form of Scrib mainly composed of the LRR domain, which is crucial for its proper localization, in addition of provoking cell cycle arrest at G1/G0 phase, it also promotes apoptosis in MDA-MB231 human breast cancer cells that bear the activated oncogenes KRAS and MYCL. Whereas, T-47D cells harboring the oncogenic PIK3CA mutation, despite the fact that the truncated Scrib trigger similar cell intracellular signaling events, does not behave the same way. Our data establish a role for c-Myc in Scrib94-494-mediated apoptosis in human breast cancer bearing activated KRAS and MYCL mutations.

According to catalogue of somatic mutations provided by cosmics, MDA-MB231 harbor mutation in ArfGAP1. Furthermore, it is proven that the expression of the LRR domain alone enhances apoptotic cell death in βPIX-knockdown MDCK cells, whereas overexpression of the LRR domain of Scrib in MDCK cells with normal levels of βPIX had no effect on cell viability (Frank et al., 2012). Taking the above into consideration, the Scrib94-494-mediated apoptotic cell death of MDA-MB231 ArfGAP1 mutated cells, may mimics the effect mediated by βPIX-deletion in MDCK cells, driving cells to apoptosis.

It has been shown that Myc induces apoptosis through Rac/JNK/c-Jun/Bim signaling cascade in a Scrib-dependent manner (Zhan et al., 2008). Consistent with this, in our RNA-seq analysis ectopic expression of Scrib94-494 demonstrated a 4-fold increase in Myc mRNA levels and si-RNA silencing of Myc rescue MDA-MB231 Scrib94-494 cells from apoptosis. Whereas in our cell context, si-RNA silencing of Myc enhances JNK/c-Jun cell signaling, implying the occurrence of a mechanism that upregulates Myc and

drives cells to apoptosis independently of JNK/c-Jun signaling axis. Pharmacological abrogation of mTORC1 signaling indicated the central role of mTORC1 in apoptosis induction.

Our results demonstrate a novel mechanism of Scrib-mediated regulation of mTOR signaling. It is proven that Scrib interacts with P90-RSK2 (ribosomal S6 kinase 2) (Zenniou-Meyer et al., 2008). P90-RSK2 is a serine/threonine-protein kinase that acts downstream of ERK (MAPK1/ERK2 and MAPK3/ERK1) signaling cascade. P90-RSK2 regulates translation through RPS6 and EIF4B phosphorylation, and mediates cellular proliferation, survival, and differentiation by regulating mTOR signaling and repressing pro-apoptotic function of BAD and DAPK1. Additionally, P90-RSK2 phosphorylates GSK3β at Ser-9 inhibiting its activity. Our data, indicate that the truncated Scrib enhances GSK-3β phosphorylation at the site of Ser-9, possibly through a mechanism Scrib94-494-mediated P90-RSK2 activation.

Proteomic analysis revealed GIT1 as a novel mTOR complex component in astrocytes (Smithson & Gutmann, 2016). Scrib it has been shown that Scrib interacts with βPIX and exists in a complex with the Arf-GAP GIT1, a known positive modulator of MEK-ERK signaling (Audebert et al., 2004, Yin et al., 2004). Taken these findings into consideration, Scrib94-494 could act as a positive regulator of mTORC1 activity, participating in a novel Scrib/β-PIX/GIT1/mTOR complex.

Our findings provide novel insights for understanding the interplay of mutated Scrib with components of signaling pathways in a breast cancer cell model in which several oncogenic signal transduction pathways are activated. Results in cell cycle arrest and apoptosis.

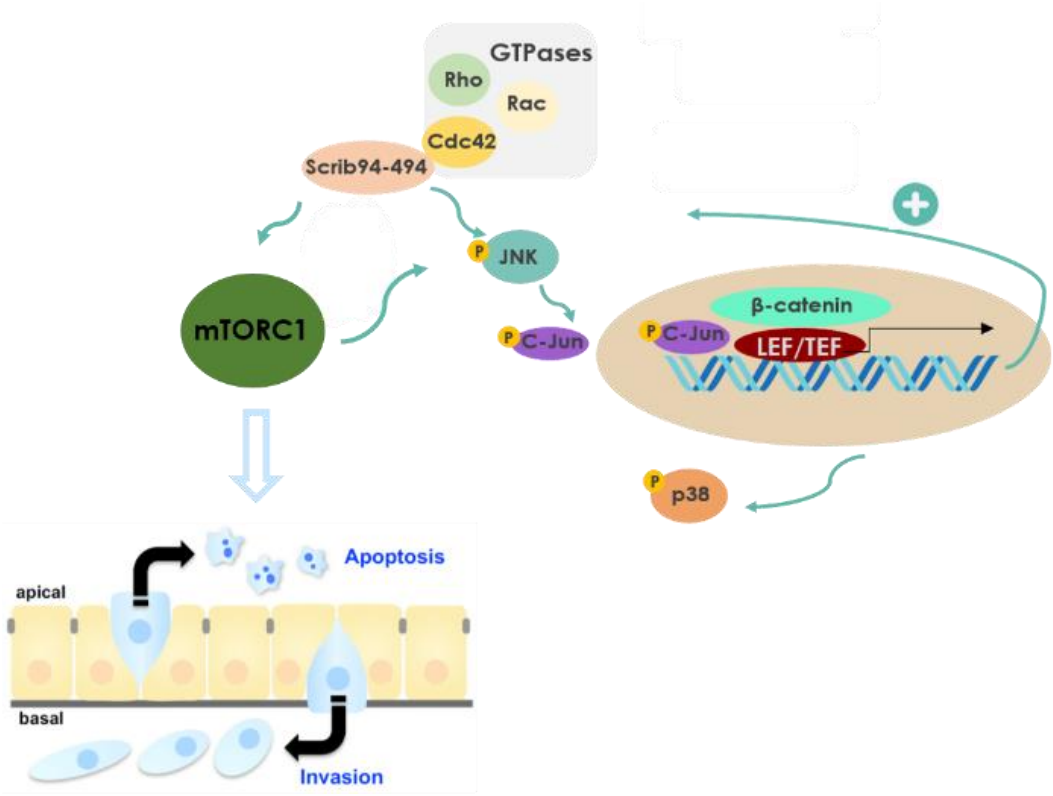


Figure 5.1. Summary of the results and a model for Scrib94-494-mediated apoptosis and cell invasion. The truncated Scrib: a) activates JNK/c-Jun and p38-MAPK signaling axes in ASK1 independent manner, b) functions in Wnt signaling regulating the phosphorylation status of GSK3β, c) activates mTORC1 signaling, independently of Akt and TSC2, d) regulates c-Myc expression via mTORC1 and β-catenin signaling, e) Inhibits the recruitment of β-catenin with CBP as well as inhibition of mTOR rescues cells from apoptosis, f) induces apoptosis and increases matrix degradation.

6. Literature

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