Cell signalling through the Wnt pathway

Inauguraldissertation
der Philosophisch-naturwissenschaftlichen Fakultät
der Universität Bern

vorgelegt von

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Von der Philosophisch-naturwissenschaftlichen Fakultät angenommen.

Bern, den 27. Mai 2004 Der Dekan: Prof. Dr. G. Jäger

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Summary

Wnt proteins control numerous aspects of development, are evolutionarily highly conserved and deregulation of their signalling activity is involved in several diseases including cancer. Wnt signal transduction proceeds through a complex series of protein interactions, initiated by binding of the Wnt protein to a cell surface receptor-complex (the transmembrane Frizzled receptor together with the co-receptor LRP) which generates a signal to downstream components. A key event in signalling is the regulation of glycogen synthase kinase 3β (GSK3β) and the phosphorylation of its substrate β-catenin. In the absence of a Wnt signal, GSK3β phosphorylates β-catenin, which then becomes targeted for degradation. The binding of Wnt to its receptors initiates a cascade of events that inhibit the kinase activity of GSK3β and ultimately prevent degradation of β-catenin. Together with the DNA binding protein TCF, β-catenin activates expression of Wnt target genes. Alternative, β-catenin-independent mechanisms of signal transduction are less well understood, but it is becoming clear that they are important for several processes related to cell movement, shape and polarity during development. The Wnt pathway also regulates several homeostatic and developmental processes occurring in adult animals, e.g. several Wnt genes are expressed during post-natal development of the mammary gland and in some cases their role in this process has been elucidated. During mammopoiesis, mammary epithelial cells undergo cycles of proliferation, differentiation and apoptosis that are regulated by a complex set of factors including hormones, cell-substratum interactions and growth factors.

In a screen for genes associated with the apoptotic phase of mammary gland development, a Wnt antagonist, now known as SFRP4 (secreted Frizzled-related protein 4) was identified in our laboratory. Furthermore, it was shown that over-expression of SFRP4 in the lactating mammary gland of transgenic mice results in increased levels of apoptosis, leading to the conclusion that SFRP4 is a factor necessary for inducing apoptosis of mammary epithelial cells. Since current apoptotic theory postulates that cells exist in a state of apoptotic potential, the progression to which is inhibited by a variety of survival signals, we postulate that SFRP4 blocks such an essential, Wnt-mediated survival signal and hence indirectly causes apoptosis. A likely mechanism by which Wnt signalling mediates survival in mammary epithelial cell is through activation of protein kinase B (PKB), which is involved in several processes that maintain cell viability. In order to address questions related to the mechanism of Wnt signalling inhibition by SFRP4 and its effect on survival we needed a model system allowing experimental

testing of our working hypotheses. Because cells in culture can readily be manipulated and fulfil the requirements of an experimental model, we decided to set up a cell culture system allowing us to modulate the pathway activity using Wnts, SFRP4 and other antagonists. The establishment of such a cell culture model involves 1) the preparation of biologically active Wnt protein, 2) the development of assays for assessing pathway activation, 3) the characterisation of the response of recipient cells to Wnt stimulation and 4) the preparation of secreted antagonists for use in inhibition experiments. The results obtained for the first three steps are presented and discussed, i.e. the stimulatory effect of purified Wnt-3a on two different cell lines has been analysed using the different assays developed for monitoring Wnt pathway activation. With respect to inhibition of the Wnt-3a-response using SFRP4 and other antagonists, no results have been obtained so far, due to our inability to inhibit the pathway even when using the ligand-independent inhibitor Dickkopf 1, which would be expected to antagonise Wnt-3a pathway activation and hence should serve as a control. Because, unsurprisingly, it appears from the literature that the ability of SFRP4 to antagonise Wnt signalling depends on the model system used, it is possible that the lack of an antagonistic effect in our cell culture model reflects a biologically relevant characteristic of this SFRP, but at the moment we can not exclude the more likely possibility of an experimental artefact. Hence, for future studies related to the role of SFRP4 in mammary epithelial cell apoptosis, it will be necessary to further develop our cell culture system and to find the conditions allowing to modulate Wnt signalling with SFRP4.

1. Introduction

1.1 The Wnt signalling pathway

The term ‘‘Wnt’’ (pronounced wint) fuses the names of two orthologous genes: *Wingless* (*wg*), a *Drosophila* segment polarity gene, and *int-1*, a mouse protooncogene[1;2]. Since the discovery of the first Wnt proteins 22 years ago, more than 2500 papers have been published documenting an impressive array of embryonic and adult processes regulated by Wnt, including cell proliferation, differentiation, survival, polarity and cell motility and resulting in the formation of embryonic axes, organogenesis and tissue homeostasis. *Wnt* genes encode a large family of secreted protein growth factors that have been identified in several species ranging from hydra to man. The transduction of Wnt proceeds in a complex series of events including post-translational modification and secretion of Wnts, their binding to a transmembrane receptor complex, subsequent activation of cytoplasmic effectors, and, finally, transcriptional regulation of target genes, thus determining cell fate. Deregulation of Wnt signalling in embryogenesis often causes lethal developmental defects[3], whereas in the adult organism it leads to tumour formation[4] and is also linked to other clinically relevant misfunctions such as Alzheimer's disease[5].

In the last years, intensive research of Wnt signalling has substantially improved our understanding of this pathway; the new insights, however, have also increased the pathway's complexity and raised many new questions. Therefore, the following description of the Wnt pathway does not offer a complete picture, but rather intends to sketch the mechanisms and aspects of Wnt signalling that are relevant to this study. Signal transduction will be outlined starting from the ligand-receptors interaction at the cell membrane (1.1.1) and then discussing the main types of intracellular pathways (1.1.2). Particular emphasis will be given to the best-understood, canonical pathway (1.1.3), which regulates target gene expression in the nucleus. The focus will then be directed on the main classes of secreted antagonists of Wnt signalling (1.1.4).

1.1.1 Wnt ligands and Wnt receptors

Wnt genes[6] have been identified in vertebrates and invertebrates, but appear to be absent from plants, unicellular eukaryotes such as *Saccharomyces cerevisiae* and from prokaryotes. In

vertebrates, 19 *wnt* genes have been identified in humans and mouse, 16 in *Xenopus*, 11 in chick, and 12 in zebrafish; in invertebrates, *Drosophila* has seven known *wnt* genes, *Caenorhabditis elegans* five and Hydra at least one[7]. Human Wnt proteins are all very similar in size, ranging in molecular weight from 39 kDa to 46 kDa. Little is known about the structure of Wnt proteins, as they are difficult to purify due to their low solubility, but all have 23 or 24 cysteine residues, the spacing of which is highly conserved, suggesting that Wnt protein folding may depend on the formation of multiple intramolecular disulfide bonds. Before being secreted, Wnt proteins undergo different post-translational modifications. One is glycosylation, which, however, might not be always important for Wnt function, since mutation of all four potential glycosylation sites in Wnt-1 does not abolish its ability to transform mammary epithelial cells[8]. A further modification that has been discovered recently is palmitoylation at cysteine 77, which corresponds to the first of the 23 invariable cysteine residues conserved among all Wnt proteins. Since enzymatic removal of the palmitate or mutation of the palmitoylation site resulted in a significant loss of bioactivity, this modification type seems to be important[9]. After secretion, Wnt proteins bind tightly to the cell surface via association with extracellular matrix (ECM) components such as proteoglycans. Little is known about the role of the ECM components in Wnt signalling, they may represent low-affinity receptors for Wnts, which would serve to increase the local concentration of ligand available for binding to high-affinity receptors, or they may also be important for the formation Wnt gradients[10].

More than ten years after the discovery of the first *wnt* genes it became clear that members of the Frizzled family function as Wnt receptors[11]. The Frizzled (Fz) receptors are named after the *Drosophila* tissue polarity gene *frizzled*, the first family member to be discovered. Meanwhile, several members of the Fz receptors family have been identified in different species; there are ten known members in humans and mice, four in flies, and three in worms. The following features characterise all Fz receptors[10]: a putative signal sequence at the N terminus, followed by a sequence of 120 amino acids containing 10 highly conserved cysteine residues, a highly divergent region of 40–100 amino acids predicted to form a flexible linker, seven transmembrane segments separated by short extracellular and cytoplasmic loops, and a cytoplasmic tail containing a consensus PDZ domain binding motif at the carboxyl terminus. The cysteine-rich domain (CRD) forms a novel protein fold with a conserved dimerisation interface that may be important for Wnt binding. The general structure of Fz receptors resembles that of seven-transmembrane G-protein-coupled receptors, suggesting that Fz proteins may use heterotrimeric G proteins to transduce Wnt signals[12]. Several recent studies provide evidence consistent with this idea, showing that a subgroup of Fz receptors can signal

through the pertussis-toxin-sensitive subclass of heterotrimeric G proteins to stimulate an increase in intracellular Ca^{2+} and activate protein kinase C (see also below)[13].

A newcomer to Wnt signalling is a single transmembrane protein that is used by Frizzled as co-receptor and that is necessary for activation of the canonical Wnt/β-catenin pathway, but does not seem to be required in the case of noncanonical Wnt signalling[14]. This co-receptor called arrow in *Drosophila* and of which there are two in vertebrates, LRP5 and LRP6, is a member of the low-density lipoprotein receptor-related protein (LRP) family. In their extracellular domain, the LRPs contain four epidermal growth factor (EGF)-like repeats and three low density lipoprotein (LDL)-receptor type A repeats. A proline-rich intracellular domain implies putative interactions with SH3-domain-containing proteins. Although the role of LRP in signalling is unclear, recent evidence suggests that binding of the cytoplasmic domain of LRP to the Wnt antagonist axin may play a role in Wnt pathway activation[15].

Considering the large number of Wnt ligands and Fz receptors, as well as the involvement of additional receptor proteins, it can be expected that individual Wnts elicit specific biological responses that are distinct from the effects of other members of the Wnt family, implying the presence of mechanisms that provide such signalling specificity[10]. One way to achieve specificity is provided by restricting the expression pattern of different Wnts (or Frizzleds) to discrete, non-overlapping regions of the organism, but there are examples for overlapping Wnt expression patterns. Another possibility is given by specificity of ligand-receptor interactions, in fact not every Wnt can bind to every Frizzled, but the contrary is also true: several cases of promiscuity in Wnt/Frizzled interactions are known. Addressing the question of Wnt-Fz interaction specificity as well as other questions regarding Wnts has been hampered by the difficulty to purify bioactive Wnts, but with the recent publication of a protocol for Wnt purification[9], advances in this research area can be expected. However, it has to be noted that *in vitro* binding experiments performed with purified proteins are prone to generate artefacts and a careful interpretation of data from such studies is crucial.

In conclusion, Wnt signalling is initiated following the binding of a Wnt ligand to a member of the Frizzled family of seven-span transmembrane receptors together with the co-receptors LRP5 or LRP6 and possibly also with the involvement of components of the ECM. An important open question relates to the mechanisms determining specificity of interaction between Wnts and Fz receptors. Distinct sets of Wnt/Fz pairs can activate distinct intracellular signalling pathways (described in the next section) leading to unique cellular responses.

1.1.2 Intracellular signal transduction

Wnt signals are transduced through different intracellular signalling mechanisms: the canonical Wnt pathway is dependent on β-catenin, whereas noncanonical Wnt signalling does not rely on it (Figure 1.1)[16]. While there is tendency to consider each intracellular transduction mechanism separately, it is important to note that these "sub-pathways" can be active simultaneously and influence each other, so that one should consider them as ramifications of a single pathway. In the intensely studied canonical Wnt/β-catenin pathway, signalling depends on the steady-state levels of the multifunctional protein β-catenin. In the absence of Wnt, β-catenin is targeted for proteasomal degradation by a destruction complex. Wnt signalling antagonises this destruction complex, leading to the accumulation of β-catenin and activation of target genes (see next section for more details). Regarding noncanonical Wnt signalling, one of the unresolved issues concerns the relevance of classifying β-catenin-independent signal transduction phenomena into discrete pathways, among which figure the Wnt/PCP (planar cell polarity), the Wnt/Ca^{2+} and other pathways. In *Drosophila*, signalling by the Wnt/PCP pathway is required for the appropriate orientation of trichomes of the adult wing and for appropriate chirality of ommatidia in the eye, and may regulate asymmetric cell divisions of neuroblasts. In vertebrates, a pathway akin to Wnt/PCP signalling controls convergent extension movements of cells during morphogenesis[17]. The Wnt/Ca^{2+} pathway plays a role in the separation of germ layers during gastrulation and in antagonising canonical signalling[18;19].

Because in the present study noncanonical Wnt signalling has not been specifically considered, in the remaining part of this section only a brief consensus view of noncanonical signalling mechanisms is presented (see also Figure 1.1). Furthermore, some of the most significant open questions in this area of Wnt research are considered[20]. Vertebrate noncanonical Wnt signalling controlling convergent extension movements requires a Fz receptor and the proteoglycan co-receptor Knypek (Kny). As in canonical signalling, Dishevelled is required, but it has to additionally be localized to the cell membrane via its DEP domain. A main branch downstream of Dishevelled involves activation of the small GTPases of the Rho family via the bridging molecule Daam1. The precise roles of Rho versus other Rho family small GTPases such as Rac and Cdc42 remain unclear, as is the potential role of the JNK pathway. Another branch involves generation of calcium fluxes and activation of kinases in a heterotrimeric G protein-dependent manner, involving activation of phospholipase C (PLC), which then hydrolyses phosphatidylinositol 4,5-biphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 catalyses the release of stored intracellular Ca^{2+}, activating

Ca^{2+}/calmodulin-sensitive kinase II (CamKII), and DAG activates protein kinase C (PKC). Until recently, the Wnt/Ca^{2+} pathway was thought to be independent of Dishevelled, however, the involvement of Dishevelled in the release of intracellular calcium has been shown, suggesting that there is a partial overlap between the Wnt/Ca^{2+} pathway and Wnt signalling controlling convergent extension movements[21;22].

Despite the many advances in understanding noncanonical Wnt signalling, many questions and controversies remain to be solved. An ever-recurring question relates to specificity of different Wnt-Fz interactions and how these determine which Wnt pathway is activated. As mentioned above, the several types of noncanonical signalling pose the question of their correct classification. Some evidence suggests that the different noncanonical pathways represent different views of the same underlying signalling mechanism; however this does not exclude the possibility that certain components are only used in particular contexts. Despite the difficulties encountered in the study to noncanonical Wnt signalling, important insights from this research field can be expected: while several signalling pathways modulate gene transcription to control cell fate, there are relatively few known to regulate directly cell movement, shape and polarity[20].

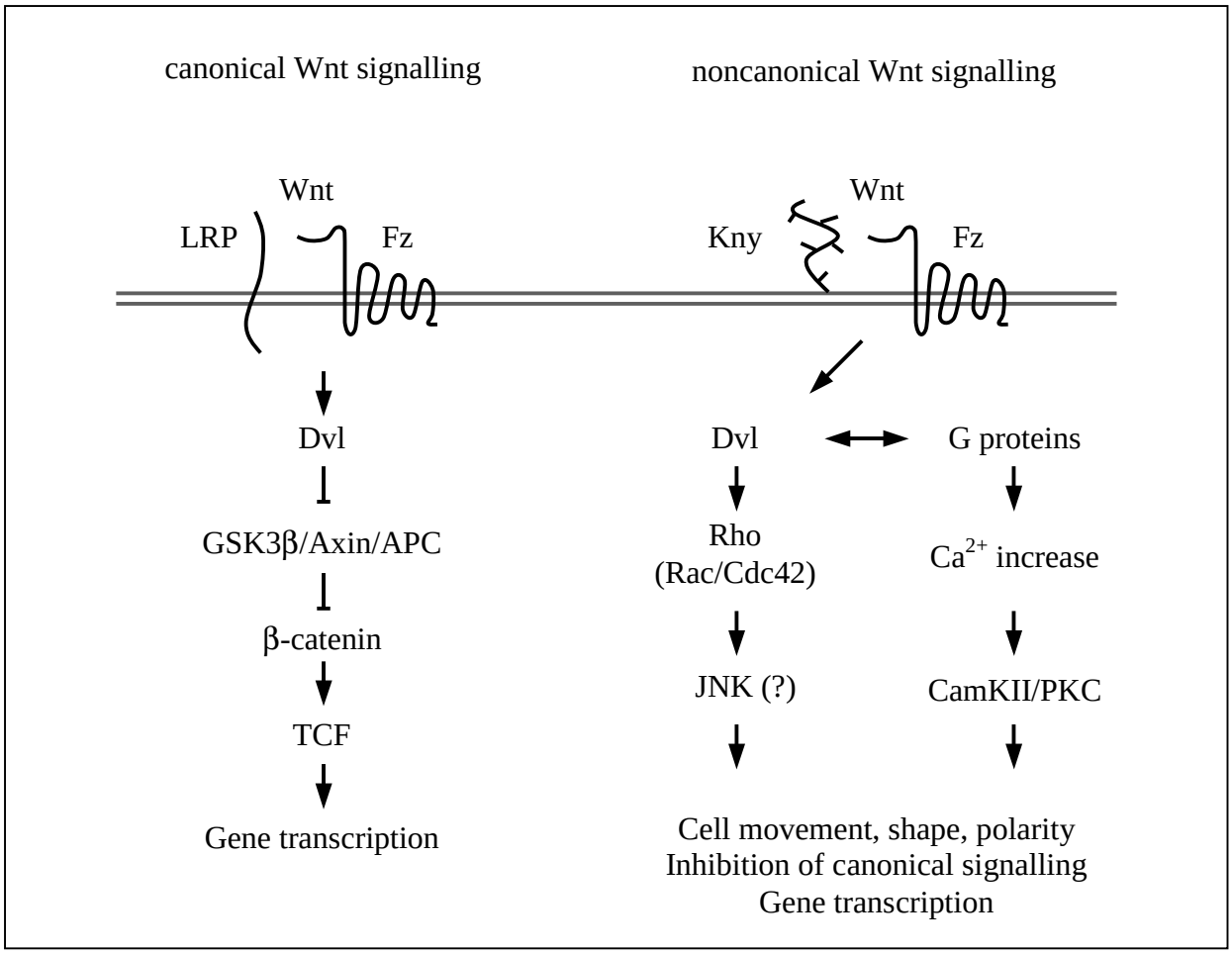

Figure 1.1 Simplified schematic description of Wnt signalling. Different types of intracellular signal transduction have been identified: the canonical Wnt pathway regulates turnover of the multifunctional protein β-catenin, noncanonical Wnt signalling is β-catenin-independent and can be subdivided into several ramifications (Wnt/Ca^{2+}, Wnt/Rho, Wnt/JNK signalling) possibly just reflecting different views of the same underlying signalling mechanisms. Arrows and blocked lines denote activation and inhibition, respectively. Abbreviations: see Appendix A.

1.1.3 The canonical Wnt signalling pathway

At the heart of canonical Wnt signalling is the multifunctional protein β-catenin (armadillo in *Drosophila*) most of which is bound to the plasma membrane, being part of the intercellular adhesion structures, the adherens junctions[2]. In the absence of a Wnt stimulus (Figure 1.2a), β-catenin is phosphorylated by casein kinase I (CKI) at Ser-45 creating a priming site which is necessary and sufficient for glycogen synthase kinase 3β (GSK3β) to subsequently phosphorylate the remaining sites of serine/threonine residues Thr-41, Ser-37 and Ser-33[23]. Phosphorylation of β-catenin occurs in a multiprotein complex containing GSK3β, the scaffold protein axin, the tumour suppressor gene product APC (adenomatous polyposis coli) and

diversin, which links CKI to the complex. Axin and APC form a structural scaffold that allows GSK3β to specifically phosphorylate β-catenin. GSK3β also phosphorylates APC and axin, increasing the stability of the protein complex. Phosphorylation of β-catenin is necessary for its binding to the F-box protein β-TrCP, a component of an E3 ubiquitin ligase complex, and for subsequent ubiquitination and degradation by the proteasome.

Upon binding of Wnt to the Fz/LRP receptor (Figure 1.2b), the β-catenin phosphorylation complex is inhibited, so that β-catenin can no longer be tagged for degradation by GSK3β. Even though it is still unclear how exactly the β-catenin phosphorylation complex is inhibited, it is clear that the cytoplasmic protein Dishevelled (Dsh in *Drosophila*; Dvl1, Dvl2 or Dvl3 in mice) is essential in this process. It has to be noted that, as mentioned above, Dvl is also responsible for transducing signals in the noncanonical Wnt pathways. Recently it has been shown, that there is a direct interaction between the PDZ domain of Dvl1 and the conserved Fz sequence that is C-terminal to the seventh transmembrane domain[24]. Furthermore, it is known that upon interaction with Wnt-activated Fz, Dvl becomes phosphorylated (probably by CKII)[25] which leads to inhibition of the activity of GSK3β. Exactly how GSK3β is regulated by Dvl is still unknown, but it may involve GSK3β binding protein (GBP), also known as Frat. Dvl has been shown to interact with axin and GBP, while GSK3β directly interacts with Axin and GBP but not Dvl. A quaternary complex of Axin, GSK3β, Dvl and GBP thus appears to exist in unstimulated cells. Following Wnt stimulation, Dvl is recruited to the membrane which is postulated to cause a conformational change allowing the GBP-mediated disassociation of GSK3β from axin and consequently disruption of the β-catenin phosphorylation complex[2]. An additional scenario could be that upon pathway stimulation Dvl activates protein phosphatase 2A (PP2A), which then dephosphorylates components of the multiprotein complex responsible for β-catenin phosphorylation, again resulting in its inhibition[26]. Finally, another mechanism leading to inhibition of the β-catenin phosphorylation complex has been suggested, involving the translocation of axin to the membrane where it interacts with the intracellular tail of LRP[15].

As a result of the Dvl-mediated inhibition of β-catenin degradation, β-catenin accumulates in the cytoplasm and eventually translocates to the nucleus by an unknown mechanism where it can activate the expression of more than seventy target genes (for an updated list see the Wnt homepage: http://www.stanford.edu/~rnusse/wntwindow.html). Because β-catenin contains a transactivation domain at the C-terminus, but does not bind DNA itself, to activate gene transcription it has to function as an essential co-factor for TCF transcription factors. The TCFs (T-cell factors) were originally identified as lymphoid-specific

transcription factors. These proteins contain a high-mobility group box that binds DNA in a sequence-specific manner, bending the DNA in the process. However, they can only activate gene transcription when bound to β-catenin, which provides transactivation activity. In the absence of a Wnt signal, TCF binds members of the Groucho (Gro) family of transcriptional repressors, which help to actively repress transcription of TCF target genes[27].

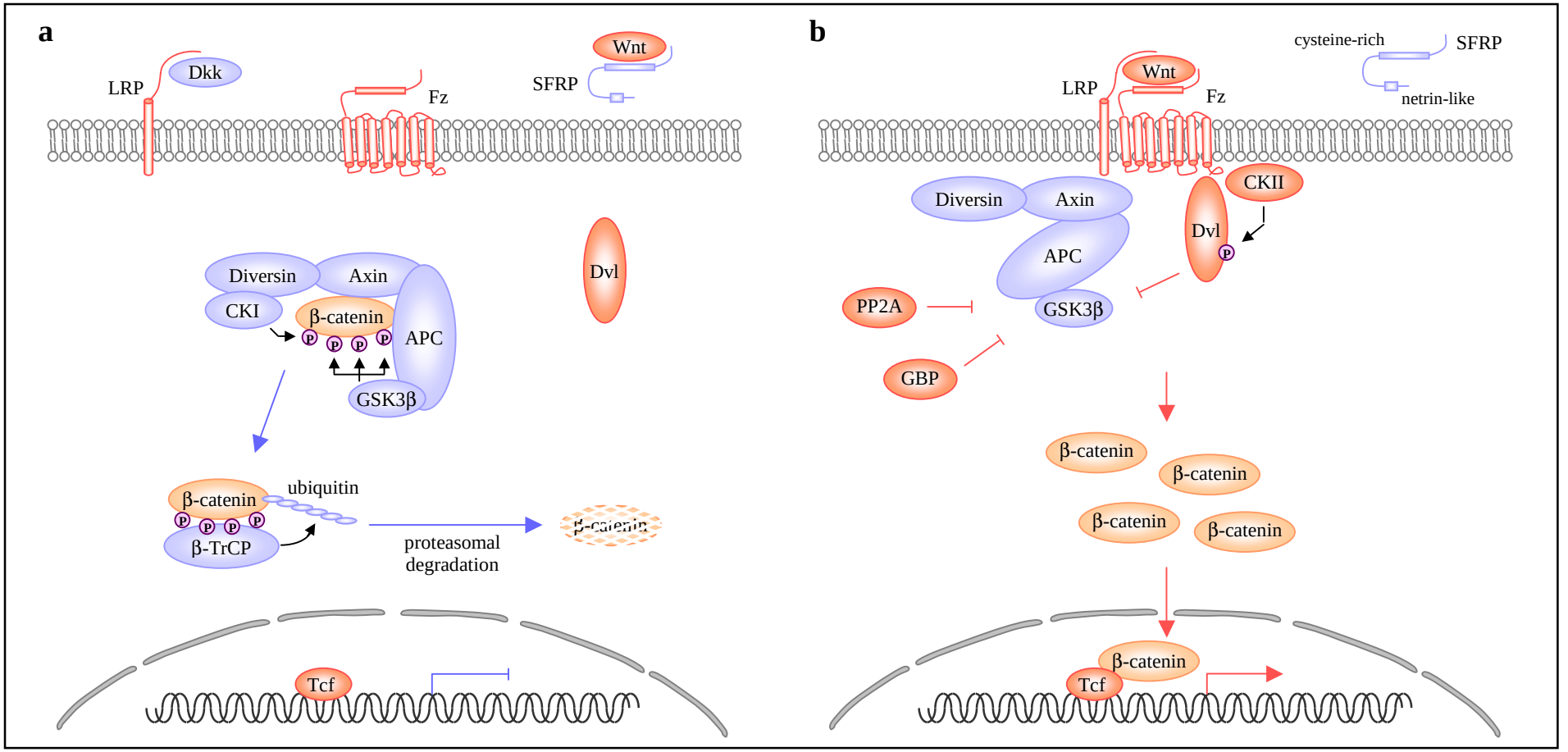

Figure 1.2 The two-state model of Wnt signalling. **a** In the absence of a Wnt ligand, the phosphorylation of β-catenin necessary for its proteasomal degradation occurs through the action of CKI and GSK3β in a multiprotein complex composed of APC, axin and diversin. **b** Upon engagement of the Fz-LRP receptor complex, β-catenin phosphorylation is inhibited by a poorly understood mechanism (involving phosphorylated Dvl, GBP, PP2A and recruitment of axin to LRP; see text for more details), resulting in accumulation and translocation of β-catenin in the nucleus, where it binds to TCF transcription factors and activates gene transcription. Pathway components negatively regulating Wnt signalling are in blue, whereas components with a positive regulatory effect are in red. Arrows and blocked lines denote activation and inhibition, respectively. Abbreviations: see Appendix A.

1.1.4 Modulation of Wnt signalling

Efficient signalling involves a tight control and modulation of the pathway. To this end, a wide range of strategies is used, including repression of transcription, inactivation of intracellular pathway components (e.g. by dephosphorylation) and secretion of regulatory or antagonistic factors. In the case of Wnt inhibition, two major functional classes of secreted modulators exist[28], which exert an inhibiting effect on Wnt signalling (see Table 1). The inhibitors of the first class, which include the 8 members of the SFRP (secreted Frizzled-related protein) family, WIF-1 (Wnt inhibitory factor-1) and Cerberus, primarily bind to Wnt proteins, thereby hindering Wnts to bind the receptor complex[29]. The second class comprises certain members of the Dickkopf (Dkk) family, which bind to the LRP co-receptor and internalise it, resulting in its removal and loss of a functional canonical signalling receptor[30].

The SFRPs share strong homology with the CRD of Fz receptors, but lack the transmembrane regions; however, they are not the product of alternatively spliced *fz* genes[31]. They were initially given several names, reflecting their simultaneous discovery by different approaches. There are presently eight known members of the SFRP family and a unifying nomenclature now exists for four of these. The first hint for the existence of secreted proteins with partial homology to Fz receptors was obtained in 1996, when SFRP2 (then called SDF-5) was discovered in a large-scale screen for secreted proteins using the signal sequence trap method[32]. One year later FRZB was identified by two independent lines of investigation, one involving the analysis of bone- and cartilage-inducing activities of bovine cartilage extracts[33], and a second aimed at detecting transcripts enriched in the gastrula Spemann's organiser of *Xenopus*[34;35]. In the same year several other investigators reported the discovery of SFRP family members: the human and murine SFRP1-4 cDNAs were cloned after a search of EST databases using Fz sequences[36]; SFRP5 was shown to be highly expressed in the retinal pigment epithelium and in the pancreas[37]; in addition, SFRP members were also detected in a screen for apoptosis-associated human genes[38]. On the basis of sequence homology, SFRP1, SFRP2 and SFRP5 form a subgroup, as do FRZB and SFRP4, which are quite distantly related to the other SFRPs. Sizzled, Sizzled2 and Crescent, which have been identified in *Xenopus* (Sizzleds) and chick (Crescent), but not in mammals, form a third homology cluster[29].

The human SFRP proteins range in size from 295 to 346 amino acids, giving polypeptides of theoretical molecular weights of 33.5-39.9 kDa. All SFRP family members possess an N-terminal signal peptide sequence comprising 20-30 amino acids rich in hydrophobic residues (particularly alanine and leucine) and potential glycosylation sites located

in the C-terminal half of the protein. In the N-terminal half of the protein lies a cysteine-rich domain, which shares 30-50% sequence similarity with the CRD of Fz proteins and include 10 conserved cysteine residues that are arrayed very like those in the Fz receptors. Towards the C-terminal regions of SFRPs are sequences that share weak sequence similarity with the axon guidance protein netrin. The netrin-like domain, which is defined by six cysteine residues and several conserved segments of hydrophobic residues, is also present in other proteins, including tissue inhibitors of metalloproteinases and some complement proteins[39].

As data about SFRPs from both *in vivo* and *in vitro* studies are accumulating, the accepted view is that SFRPs are mainly inhibitors of Wnt signalling, but that they might also be able to potentiate Wnt signalling. The expression patterns of several SFRPs have been examined in developing chick and mouse embryos[40;41]. One emerging theme is the potential of SFRPs to act in the control of morphogenetic gradients of Wnt signalling activity, i.e. to generate zones of active Wnt protein in regions where a Wnt is expressed uniformly. For example, SFRP1 and Wnt8 display overlapping expression patterns during mouse heart morphogenesis[42] and there is also evidence for opposing gradients of SFRP1 and SFRP3 expression in the developing mouse telencephalon[43]. In order to understand the significance of such expression patterns, it is necessary to find out how exactly SFRPs interact with Wnts and to know to which Wnt each SFRP can bind. To date conflicting results concerning the mechanism of SRFP-Wnt interactions are apparent: one study suggests that the CRD of SFRP is necessary for functional interactions with Wnts[44], whereas another investigation shows there is no such requirement[45]. Furthermore, the potential for the CRD to dimerise has been recognised based on crystallographic data[46], confirming an earlier study reporting that a truncated Fz receptor(Hfz6-CRD) and SFRP1 can form a complex via their homologous CRD regions[47]. Thus, SFRPs may block Wnt signalling not only by sequestering Wnts and so preventing a ligand-receptor interaction, but also by binding to Fz proteins via CRD dimerisation resulting in the formation of non-functional receptor complexes. Tissue culture experiments have shown that, at low concentrations, SFRP1 potentiates Wg activity rather than inhibiting it[45], suggesting that SFRP1 has Wnt binding sites with different affinities and that this influences the outcome of SFRP-Wnt interactions. An alternative hypothesis is that, SFRPs act as antagonists only at sites where there are few receptors by sequestering Wnts and as stimulating factors by transporting Wnts to sites that have a high concentration of receptors, where they can be released as active ligands.

Other than the SFRPs, the Dickkopf family members regulate signalling by binding the LRP co-receptor. Thus, at least in theory, Dkks specifically inhibit the canonical pathway. The

Dkk family comprises four members (Dkk1 to Dkk4) and a unique Dkk3-related protein named Soggy. Dkks contain two characteristic cysteine-rich domains (Cys-1 and Cys-2) separated by a linker region of variable length. Cys-2 is highly conserved among all members of the family and contains 10 conserved cysteine residues; this is similar to the proteins of the colipase family, with which Dkk proteins share weak sequence similarity[30;48]. During development, Dkk1, the most studied family member, functions as a head inducer secreted from the vertebrate head organiser. Injection of Dkk1 mRNA into *Xenopus* embryos results in anteriorisation with an 'enlarged head' phenotype, whereas injection with an anti-Dkk1 antibody causes a lack of anterior head structures[48], which is also the phenotype of Dkk1-knockout mice[49]. In addition to LRP5/6, Dkk1 interacts with another class of receptor, the single-pass transmembrane proteins Kremen (Krm). Dkk1, Krm and LRP form a ternary complex that disrupts Wnt signalling by promoting endocytosis and removal of the LRP co-receptor from the plasma membrane[50]. To date, the Wnt antagonist activity of Dkk4 appears to be indistinguishable from Dkk1, Dkk3 and Soggy have no effect on Wnt signalling, whereas Dkk2 can both inhibit and activate Wnt signalling depending on the experimental context[30;51-53].

Table 1. Wnt antagonists (adapted from ref.[28])

Name	Alternative names	Accession nos.	MW*	Mechanism of action	Wnt antagonist activity	Agonist activity
SFRP1	sFRP-1, FRP, SARP2, FrzA	NM_003012 (h) NM_013834 (m)	35.3 kDa 313 aa	Binds Wnt and Fz	Yes[45;54;55]	Agonist at low concentrations[45]
SFRP2	SDF-5, sFRP2, SARP1	XM_050625 (h) NM_009144 (m)	33.5 kDa 295 aa	Binds Wnt	Yes[40]	
FRZB	sFRP-3, FrzB, Frzb-1, Fritz	NM_001463 (h) NM_011356 (m)	36.2 kDa 325 aa	Binds Wnt	Yes[34;35;44;56;57]	
SFRP4	DDC-4, sFRP-4, frpHE, FrzB-2	NM_003014 (h) NM_016687 (m)	39.9 kDa 346 aa	Binds Wnt	Yes[58;59]	
SFRP5	sFRP5 (SARP3)	NM_003015 (h) NM_018780 (m)	35.6 kDa 317 aa	Binds Wnt	Yes[60]	
Sizzled**		AF059570 (x)	31.8 kDa 281 aa (x)	Binds Wnt (?)	Yes[61] No[62;63]	
Sizzled2**		Not found		Binds Wnt (?)	No[62]	
Crescent**	frzb2	AF136183 (x) NM_205099 (ch)	33.5 kDa 295 aa (x)	Binds Wnt	Yes[64]	
WIF-1		NM_007191 (h) NM_011915 (m)	41.5 kDa 379 aa	Binds Wnt	Yes[65]	
Cerberus**		NM_203515 (x)	31.2 kDa 276 aa (x)	Binds Wnt	Yes[66]	
Dkk1	DKK1	NM_012242 (h) NM_010051 (m)	28.7 kDa 266 aa	Binds LRP5/6	Yes[30;48;51;67;68]	Inhibits Dkk2[68]
Dkk2	DKK2	NM_014421 (h) NM_020265 (m)	28.5 kDa 259 aa	Binds LRP5/6	No (in Xenopus embryos)[30;68] Yes(in Xenopus embryos)[51] Yes (in cell lines)[52;68]	Agonist[68]Agonist with LRP6[52;52]
Dkk3	REIC, DKK3	NM_013253 (h) NM_015814 (m)	38.3 kDa 350 aa		No[30;53]	
Dkk4	DKK4	NM_014420 (h) NM_145592 (m)	24.9 kDa 224 aa	Binds LRP5/6 (?)	Yes[30;53]	
Soggy	DKKL2	NM_014419 (h) NM_015789 (m)	27.0 kDa 242 aa		No[30]	

Abbreviations: ch, chick; h, human; m, mouse; x; *Xenopus*

* MW as predicted from chain length (aa) and amino acid composition of the protein (human except where indicated)

** To date, mammalian genes for Sizzled, Sizzled2 and Crescent have not been identified. The mammalian gene related to Cerberus (mCer1) does not encode a Wnt antagonist

1.2 Mammary gland involution and apoptosis induction by SFRP4

Our interest in the Wnt signalling pathway and in its inhibition arose when SFRP4 was identified during a screen for genes involved in involution of the mammary gland in force-weaned mice[69]. Further investigations showed that the SFRP4 expression is necessary to start the apoptotic process during mammary gland involution[70], but how exactly this is achieved needs to be investigated further. In the following sections, the mammary gland as a model for the study of apoptosis will be presented (1.2.1) and then the data concerning the detection and involvement of SFRP4 in mammary gland involution will be summarised (1.2.2). Next, factors necessary for the survival of mammary epithelial cells will be described (1.2.3) and finally the involvement of protein kinase B (PKB) and Wnts in survival signalling will be considered (1.2.4).

1.2.1 Post-natal development of the mammary gland

The mammary gland, which appeared with the mammals 200 million years ago, presents a unique feature compared to the other body organs: it develops predominantly in the postpartum mammal[71]. Mammopoiesis is dependent on epithelial-mesenchymal interactions and on hormonal signals and progresses gradually attaining full functional differentiation only when required by the birth of young. The mammary gland consists of two main constituents: the ectodermal parenchyma and the mesodermal adipose stroma. The parenchyma is composed of secretory and ductal epithelium, contractile myoepithelium and stem cells[72], and it develops within the adipose stromal tissue. The rudimentary mammary epithelial anlage is laid down as an epidermal invagination during embryonic development. At birth, the stromal adipose tissue (called fat pad) contains few epithelial ducts that emerge from the nipple forming a branching ductal tree[73]. With the onset of ovarian function (estrogen production) during puberty an accelerated extension and branching of the mammary ducts takes place and large club-shaped terminal end buds (TEBs) appear at the end of the growing ducts. The TEBs consist of two distinct cell types: the body cells, which are the precursors of mammary epithelial cells (MECs), and the cap cells, which give rise to the myoepithelial cells. At the end of puberty, the fat pad is filled with a network of ducts and the TEBs have disappeared[71]. When full sexual maturity is reached, the mammary gland begins to be subjected to cyclic phases of growth, differentiation and regression during the oestrous cycle[74], but it is only upon demand during pregnancy and lactation that a full functional differentiation occurs. During pregnancy, the elevated levels of

estrogen and progesterone cause additional ductal branching and formation of lobulo-alveolar structures, i.e. the milk-secreting units[75]. At parturition, the mammary fat pad is completely filled with lobules full of alveoli and milk secretion is induced by a complex interplay of factors, including the suckling stimulus and the changing levels of progesterone and prolactin. Lactation is maximal by 10 days post-partum and is maintained for about three weeks after parturition[76]. After weaning of the young, the mammary gland enters the regression and remodelling phase of development, called involution, which serves to restructure the mammary gland in preparation to the next pregnancy. The process of involution comprises a massive epithelial cell death by apoptosis, proteolytic degradation of the extracellular matrix and global tissue remodelling. The first stage of involution, which lasts for approximately 72 hours and is reversible up to about 48 hours, is initiated by removal of the suckling stimulus and is characterised by apoptosis of the alveolar epithelial cells; in the second stage of the involutive process, the remodelling phase, tissue proteinases are induced and the basement membrane and the extracellular matrix are degraded. At the end, most of the secretory mammary epithelium has undergone apoptosis and phagocytic removal. The regressed mammary gland consists mostly of a fat pad surrounding a branched system of ducts with few alveoli left, resembling a pre-pregnancy gland of a virgin mouse[77-79]

Concerning the regulation of mammary gland development, it occurs both at a systemic level through the action of hormones and at a local and cellular level through cell-cell and cell-ECM interactions. Although distinct morphogenetic roles have been assigned to systemic hormones and local growth factors (including Wnts), the cascades and networks of signals leading to a cellular response and subsequent activation of developmental programs are in many cases poorly understood and have been reviewed elsewhere[73;80;81]. Relevant for the present study, are regulatory processes involved in the apoptosis of mammary gland epithelial cells and they will discussed in section 1.2.3. In conclusion to this section about mammopoiesis, it is important to mention the advantages and importance of using the mammary gland as a model for the study of development and apoptosis. Because the mammary gland develops predominantly in the postpartum mammal, an entire developmental program, mimicking embryonic development of other organs, can be viewed and followed in postpartum animals. As a consequence, the tissue can be easily manipulated, and reasonable amounts of tissue are available for analysis. Furthermore, because the epithelium penetrates the fat pad only in pubertal animals, it can be removed surgically before this stage and a small piece of epithelium from another animal can then be implanted into the ‘‘cleared’’ fat pad, where it will develop. In this way it is possible to generate chimeric glands composed of tissues from transgenic and

wild-type animals[71]. Apoptosis of MECs occurs not only during involution, but also during development and during the rounds of proliferation and regression of mammary epithelium which take place in the oestrous cycle. Hence, apoptosis represents a physiological process that functions to eliminate excess, damaged or defective cells during organ development and homeostasis. Dysfunctions in this mechanisms can lead not only to developmental defects, but also to different adult pathologies, in particular to mammary tumours. It follows that an understanding of processes of mammary gland development and involution is necessary to improve our understanding of breast cancer, one of the most frequently diagnosed malignancies among women of the Western world.

1.2.2 SFRP4 in the mammary gland of normal and transgenic mice

Because during mammary gland involution, genes involved in the remodelling process are up-regulated concomitantly with genes implicated in apoptosis, it is challenging to detect apoptosis-associated genes. To overcome this difficulty, the pattern of gene expression in the involuting mammary gland can be compared to the pattern observed during involution of the prostate following castration, where no tissue remodelling occurs. For this purpose, a coincidence analysis based on the RNA differential display method has been used to identify genes expressed in both models of apoptosis and five clones showing coincident expression during apoptosis in mammary and prostate tissues were isolated[82]. One of these clones, called differential display coincidence 4 (DDC-4), was analysed further[69] and was shown to be the rat homologue of murine SFRP4, which had been described few months earlier in a screen for SFRPs[36]. The expression pattern of SFRP4 has been studied further in the ovary after ovulation and in the post-partum corpus luteum, also subjected to apoptosis-mediated tissue regression[83;84]. These studies corroborate the association of SFRP4 with apoptosis, but they can not determine whether SFRP4 is a factor causing apoptosis or whether apoptosis up-regulates its expression. This question has been addressed using a transgenic mouse model over-expressing rat SRFP4[70]. The transgene expression was put under the control of the MMTV-LTR (mouse mammary tumour virus long terminal repeat) promoter, which is active in the mammary gland in late pregnancy and early lactation, a time when no endogenous SFRP4 is expressed. The mice from the two transgenic lines generated showed a phenotype that reflects a premature onset of apoptosis in the lactating mammary gland. First, transgenic mothers show lactational insufficiency as evidenced by the reduction of litter size and of offspring weight (fostering of the young by a normal mother resulted in normal weight gain). Secondly, detection of DNA

fragmentation by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling) and detection of active, cleaved caspase-3 showed that many more alveolar cells in transgenic mammary glands were apoptotic between late pregnancy and early lactation as compared to normal glands. Finally, transgenic glands at day 1-2 of lactation exhibited a significant increase in caspase-3 activity as compared to corresponding normal mammary glands. These data show a causal relationship between SFRP4 expression and induction of apoptosis; however, the precise mechanism of apoptosis induction by ectopic expression of SFRP4 remains to be defined. Analysis of the protein kinase B (PKB, also known as Akt) and several of its substrates, which are important factors regulating cell viability, offers evidence for a connection between the activity of SFRP4 and the abrogation of survival signals. Compared to the normal mammary gland, in the transgenic glands, phosphorylation and kinase activity of PKB was reduced, as was the case for the phosphorylation levels of the transcription factor forkhead, of GSK3β and of BAD. These proteins are pro-apoptotic factors that are inactivated by PKB-mediated phosphorylation. The role of PKB and of other factors implicated in survival of MECs and the potential links to the Wnt pathway will be discussed below. In conclusion, the evidence collected in the past years in the context of SFRP4 expression in tissue regression shows that SFRP4 is necessary for the onset of the apoptotic process. As for its exact role, it is hypothesised, that inhibition of the Wnt pathway by SFRP4 causes the abrogation of survival signalling, which is discussed in the next section.

1.2.3 Survival signalling in mammary epithelial cells (MECs)

At the end of the lactation period, a lack of suckling causes milk stasis within the alveolar lumen and a drop in the level of lactogenic hormones is observed. When lactation is stopped abruptly by pup removal or teat-sealing, a widely used experimental procedure, loss of systemic hormone stimulation is not necessary for induction of involution. It appears that the physical strain caused by the milk-engorgement of the alveoli is sufficient for initiation of the first, reversible stage of involution, the induction of apoptosis of alveolar cells[85]. However, it is not clear which mechanisms are responsible for initiation of the apoptotic program and for the change of gene expression patterns observed during the transition from lactation to involution. The current view is that a progressive loss of survival signals that counteract a default program of apoptosis causes MECs to die. Survival signals for MECs are provided by a signalling network formed by hormones, growth factors, cell-cell and cell-ECM interactions (Figure 1.3). An example for the interaction of different signalling pathways in mammary gland function, is

the expression of β-casein, which is dependent not only on prolactin release from the pituitary gland, but also on anchorage of the epithelial cells to the basement membrane via integrin-laminin interactions. In addition to its regulation of gene expression via Stat transcription factors, prolactin can also promote cell survival by activating the phosphoinositide 3-kinase (PI3K) pathway. Upon binding of prolactin to its receptor (PRLR), the Src-like kinase Fyn is activated and phosphorylates Cbl, an adapter protein that interacts with and activates PI3K[86]. A downstream target of the PI3K pathway and central player in survival signalling is the serine-threonine kinase PKB, whose survival-promoting proprieties are discussed below.

Concerning the role of other hormones in survival of MECs, it was shown that locally administered glucocorticoid and progesterone inhibit apoptosis of MECs in the involuting mammary gland. This inhibition was paralleled by a strong inhibition of stromelysin-1 and sulfated glycoprotein-2, which are normally expressed during mammary gland involution and it has been suggested that this is caused by impairment of the transcriptional activity of AP-1 (activator protein-1)[87]. As to the ovarian steroid estrogen, its mitotic role in cell proliferation during mammary gland development and tumourigenesis is well documented[88], but questions remain about the precise role of estrogen in promoting MEC viability. Due to the important antiapoptotic effects of PKB, it is interesting to note that it can be activated by estrogen. The ligand-stimulated estrogen receptor α can interact with the p85α regulatory subunit of PI3K, thereby stimulating it and leading to the activation of PKB[89]. Furthermore, it was shown that this estrogen effect can be potentiated, when estrogen synergies with insulin-like growth factor I[90].

In the context of MEC survival, insulin and insulin-like growth factors (IGF) are also important and inhibition of their effects was shown to be involved in apoptosis during mammary gland involution. Insulin/IGF signalling is complex in that it is mediated by three ligands (insulin, IGF-I, IGF-II) and five types of receptors and it is modulated by six IGF binding proteins (IGFBP). The intracellular responses are as diverse as glucose transport and metabolism, gene transcription and cell cycle regulation and mediated by different pathways, including the MAPK and PI3K/PKB pathways. With respect to mammary gland involution, transgenic mice over-expressing IGF-I under the control of the WAP (whey acidic protein) promoter showed a lower level of apoptosis during mammary gland involution[91]. A similar phenotype of reduction in mammary epithelial apoptosis has been observed in transgenic mice over-expressing IGF-II under the control of the MMTV-LTR promoter and expression of the transgene correlated with increased phosphorylation of PKB[92]. On the contrary, mammary

glands from transgenic mice over-expressing IGFBP-5 before involution, when IGFBP-5 is normally expressed, displayed premature apoptosis, a phenotype reminiscent of the one observed in SFRP4 over-expressing mice[93]. Addition of IGFBP-5 to cells in culture causes a block in PKB phosphorylation, providing evidence that this protein regulates apoptosis by inhibiting the insulin survival signals mediated by PKB[94]. Finally, several studies (reviewed in ref.[81]) show that growth factors of the epidermal growth factor (EGF) family and of the fibroblast growth factor (FGF) family are also involved in survival of MECs.

The survival factors described so far can be grouped in the class of soluble factors; another important class of survival signals is related to the dependence of most cells to adhere to adjacent cells and to the ECM. In culture, MECs have a specific requirement for a basement membrane rich in laminin for survival and undergo apoptosis on other types of ECM such as collagen I or fibronectin[95]. Cell anchorage is mediated by integrins, a major family of heterodimeric transmembrane receptors that link components of the ECM with the cytoskeleton. The binding of integrins to ECM components leads to their clustering and to formation of focal adhesions, which are multiprotein complexes containing architectural proteins (α-actinin, talin vinculin, tensin) and signalling proteins, including focal adhesion kinase (FAK), integrin-linked kinase (ILK) and components of the growth factor signalling pathways[96]. FAK is a non-receptor protein tyrosine kinase that becomes activated by autophosphorylation when recruited by integrin clustering to a focal adhesion complex. Active FAK can recruit and activate other signalling proteins, one of which is PI3K[97]. ILK, which is also regulated in response to adhesion, contributes to survival signalling by inhibiting the pro-apoptotic GSK3β and by stimulating the activity of the anti-apoptotic PKB[98]. Both classes of survival signals, the soluble factors and the anchorage-dependent signals, activate signalling pathways that can cross-talk with each other and thereby form a signalling network. A central player in this signalling network is PKB, which plays a critical regulatory role in diverse cellular processes and is considered an important mediator of survival. Furthermore, both the Wnt pathway and PKB inhibit GSK3β and there is evidence for a role of Wnts in activating PKB. In the next section, the mechanisms used by PKB for promoting survival and the role of Wnt signalling in the context of apoptosis prevention will be discussed.

1.2.4 The role of PKB and Wnt in survival signalling

A key regulator of PKB is the lipid kinase PI3K which is activated when recruited to the plasma membrane by a number of signals including those generated by insulin, IGF and other growth factors and by attachment to the ECM[99;100]. At the membrane, PI3K converts the glycerophospholipid phosphatidylinositol 4,5 biphosphate (PIP_2) to phosphatidylinositol 3,4,5 triphosphate (PIP_3), a process reversed by the major PIP_3-phosphatase PTEN. PIP_3 mediates translocation of PKB from the cytosol to the plasma membrane by interacting with the N-terminal PH domain of PKB. At the plasma membrane, PKB is activated by phosphorylation through PDK1 (3-phosphoinositide dependent protein kinase 1), so that it then dissociates from the membrane and starts phosphorylating its substrates, several of which have been described[101]. With respect to its ability to promote cell survival, PKB inhibits proteins that mediate apoptosis by directly phosphorylating them (see also Figure 1.3). The first of these substrates to be identified was the Bcl-2 family member BAD, which promotes apoptosis by interacting with Bcl-X_L on the mitochondrial membrane. Phosphorylation of BAD by PKB leads to its interaction with the 14-3-3 protein, which prevents it from binding to Bcl-X_L and thereby suppresses apoptosis[102]. Other pro-apoptotic factors that can be inhibited by PKB are caspases, key executioners of apoptosis, several of which possess putative PKB-phosphorylation sites. Human caspase-9 is phosphorylated by PKB at the Ser196 residue, however, this residue is not conserved in monkey and rodent homologues, making it unlikely that phosphorylation of caspase-9 is a key mechanism regulating apoptosis[103]. Other direct targets of PKB involved in apoptosis are members of the forkhead (FKHR) transcription factors family. Dephosphorylated forkhead translocates to the nucleus and induces transcription of the pro-apoptotic Fas ligand; however, PKB-phosphorylation results in the retention of forkhead in the cytoplasm and consequently abrogation of transcription[104]. PKB has also been implicated as a signalling intermediate upstream of survival gene expression that is dependent on the nuclear factor kappa B (NF-κB). The inhibitor of NF-κB protein (IκB) sequesters the ubiquitous, heterodimeric transcription factor NF-κB in the cytoplasm. However, upon phosphorylation, IκB becomes targeted for ubiquitination and is degraded by the proteasome, so that NF-κB is free to translocate to the nucleus and to subsequently activate target genes. IκB phosphorylation is regulated by a protein complex that includes three subunits IKKα, IKKβ and IKKγ/NEMO (IκB kinases), which in turn is activated by a variety of stimuli, including cytokines, activated antigen receptors and growth factors. PKB has been shown to activate the IKK complex when stimulated by PDGF[105] and other factors[106;107]; however, the molecular details of how PKB

regulates the IKK complex remain to be characterized. Likewise, the NF-κB target genes important for survival need to be characterised further, but include the pro-survival Bcl-2 family member Bfl-1/A1 and the caspase inhibitor IAP (see ref.[108]and references therein).

The first substrate of PKB to be described was GSK3, when PKB was identified as the kinase mediating the effects of insulin on cellular metabolism[109]. GSK3 is a serine/threonine kinase, of which an α and a β isoforms are known, that was first isolated and purified as an enzyme capable of phosphorylating and inactivating the enzyme glycogen synthase. We now know that GSK3β is an important component of signalling systems coupled to receptors for insulin, a variety of growth factors and other signalling molecules. In the context of Wnt signalling, GSK3β was described above as a key player in phosphorylation of β-catenin leading to degradation of the latter. GSK3β exerts several of its functions by regulating a number of other transcription factors including AP-1, CREB, heat shock factor-1 and NF-κB[110]. Concerning its role in cell survival, it has become clear that GSK3β is an important modulator of apoptosis, even though the exact mechanisms by which GSK3β facilitates apoptosis have not yet been defined precisely. Transient overexpression of active GSK3β in PC12 and Rat-1 cells was found to be sufficient to induce apoptosis, whereas expression of catalytically inactive GSK3β reduced apoptosis induced by an inhibitor of PI3K[111]. Furthermore, it was shown that relatively low levels of GSK3β overexpression, which alone did not induce apoptosis, greatly facilitated pro-apoptotic signalling activities[112]. Collectively, these and other studies leave little doubt that GSK3β is a critical intermediary in a variety of apoptotic signalling pathways and that the inhibitory control of GSK3β by anti-apoptotic signalling systems, such as the PI3K/PKB pathway, is an important mechanisms used to promote cell survival. A peculiarity of GSK3β as compared with other protein kinases is that it is normally active in cells and that it is primarily regulated through inhibition of its activity. Inactivation of GSK3β occurs, when an inactivating kinase such as PKB, $p90^{RSK}$ or PKC phosphorylates GSK3β on serine 9. The thus phosphorylated N-terminus becomes a pseudo-substrate that occupies the substrate binding pocket and active site of GSK3β, thereby preventing the binding and phosphorylation of any true substrate[113].

Because GSK3β is also inhibited upon exposure of cells to Wnts, an important question is weather the PI3K/PKB and the Wnt pathway cross-talk at the level of GSK3β or if there exists some mechanism preventing interference between the two pathways. Wnt-mediated inactivation of GSK3β is not yet understood completely, but seems to involve both

phosphorylation of GSK3β and complex formation with GBP. Upon Wnt stimulation, activated Dvl recruits GBP and forms a complex with axin and GSK3β, this leads then to GBP-mediated dissociation of GSK3β from axin, resulting in a block of β-catenin phosphorylation[114]. Concerning the role of PKB in the regulation of GSK3β activity, inhibition by PKB-phosphorylation on serine 9 has not been observed upon Wnt stimulation[115]; however, it has been suggested that PKC might be involved in the inhibition of GSK3β in the context of Wnt stimulation[116;117]. According to this evidence, the function of GSK3β in the Wnt pathway appears to be insulated from regulatory mechanisms that lie outside of the Wnt pathway. How this insulation occurs is unclear, but it probably stems from the effective sequestration of a fraction of GSK3β with axin in the destruction complex. However, this sequestration does not seem to be sufficient to completely prevent cross-talk between the Wnt pathway and PKB. Other than in the study mentioned above, where a short Wnt stimulation was used, prolonged or constitutive Wnt treatment resulted in activation of PKB and consequent serine 9 phosphorylation of GSK3β and concomitant accumulation of β-catenin[118]. Confirmation of this connection between Wnt and PKB comes from the observation that transgenic mice over-expressing SFRP4 and therefore probably having reduced Wnt activity also have a reduced PKB activity[70]. Evidence corroborating this hypothesised link between the Wnt pathway and PKB has been obtained using 3T3-L1 preadipocytes ectopically expressing Wnt1. Upon serum withdrawal, Wnt-1 causes 3T3-L1 cells to resist apoptosis through a mechanism that was shown to depend on Wnt-1-induced expression of among others *IGF-I* and *IGF-II*. Consistent with IGFs mediating the anti-apoptotic effect, the observed increase in PKB phosphorylation upon Wnt1 stimulation was hindered by addition of recombinant IGFBP-4[119]. Another mechanism for Wnt-mediated activation of PKB is through WISP-1 (Wnt1-induced secreted protein), which has been shown to lead to Ser473 phosphorylation of PKB and to have anti-apoptotic effects[120]. Hence, it appears that the activation of PKB by the Wnt pathway occurs indirectly through expression of anti-apoptotic genes, some of which act on PKB. However, at least theoretically, also a direct Wnt-induced stimulation of PKB can be hypothesised. Because several compounds acting through G protein-coupled receptors were shown to be able to activate PKB[121], it is possible that PKB is directly activated by a Wnt stimulus via a noncanonical Wnt pathway. Other studies demonstrate the anti-apoptotic role of the Wnt/β-catenin pathway in a variety of cell and tissue types[122-125]. In the case of the mammary gland, increased levels of apoptosis were observed in experiments involving expression of a dominant negative form of β-catenin both in the mammary gland of transgenic animals and in the mammary cell line HC11, showing that

functional Wnt/β-catenin signalling is required for survival of MECs[126]. However, in this study, no evidence for a role of PKB in mediating the anti-apoptotic effect of Wnt signalling could be found. Hence, even though evidence for an anti-apoptotic role of Wnts is accumulating, the precise molecular mechanisms linking this pathway to the survival signalling network remain to be defined.

In conclusion, according to what was described above, our working hypothesis is that the Wnt pathway participates in the maintenance of cell survival probably by regulating PKB and/or other anti-apoptotic factors. We further hypothesise that, at the onset of the involutive process in hormone-dependent tissues, expression of SFRP4 results in its binding to a Wnt, inhibition of signalling and induction of apoptosis.

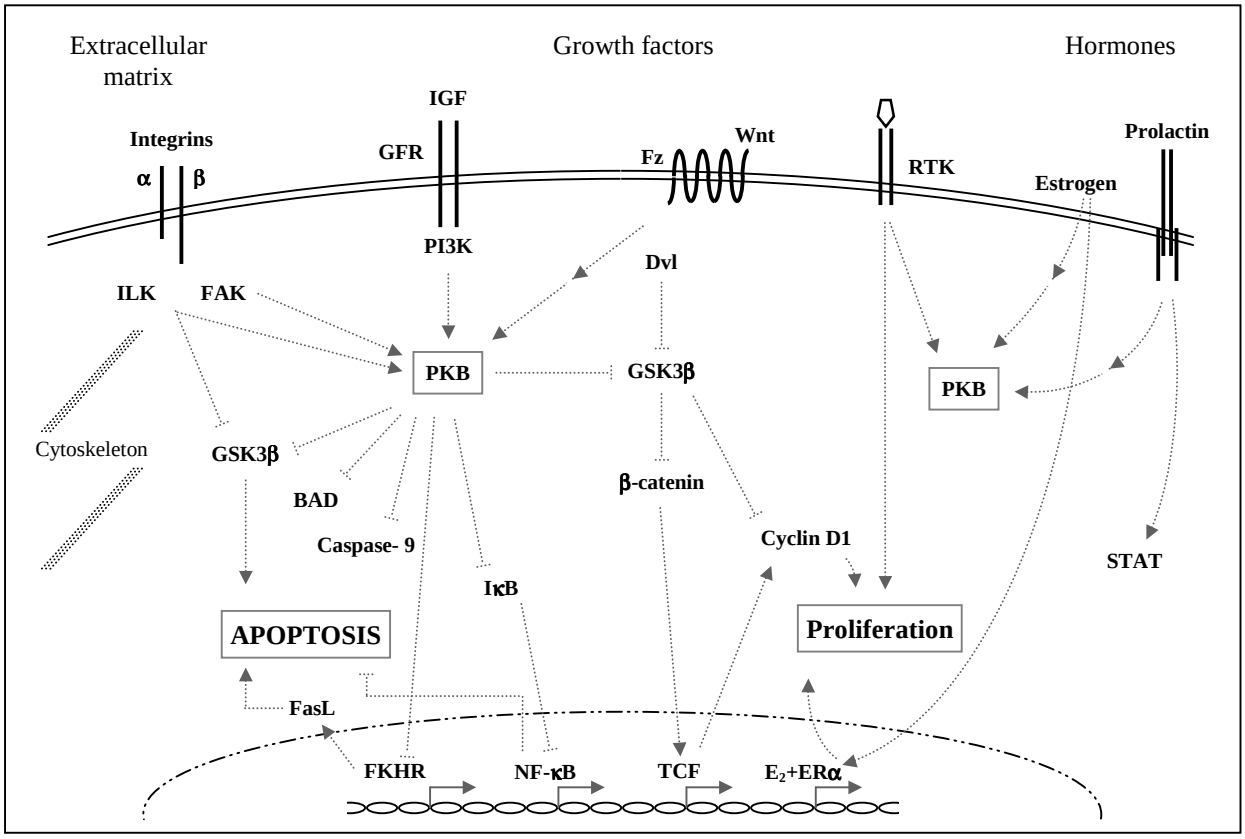

Figure 1.3 Signalling pathways important for survival of mammary epithelial cells. In this scheme of the survival signalling network active in the mammary gland some of the aspects described in the text are shown; however, it has to be noted that it represents a simplified view of the pathways and their interactions found in a (mammary epithelial) cell. In particular, for none of the shown components the full repertoire of effects could be depicted. It has to be noted further that PKB (boxed) has been depicted twice for ease of representation and not because two pools of PKB are present. Arrows and blocked lines denote activation and inhibition, respectively. Abbreviations: see Appendix A.

1.3 Open questions and study objectives

The prototype member of the Wnt gene family, *wnt-1*, was first isolated as a common site of integration by the mouse mammary tumour virus (MMTV) and its expression was shown to be up-regulated in MMTV-infected glands, resulting in the formation of mammary tumours[1]. However, in spite of the intense investigations that followed these discoveries, our understanding of the requirement for Wnt signalling in the mammary gland is still fragmentary. In the following sections, the most important open questions related to the role of Wnt signalling during mammary gland involution will be considered (1.3.1) and then the objectives of the present study will be formulated (1.3.2).

1.3.1 Open questions

Several *wnt* genes are expressed during the different developmental stages of the mammary gland. *Wnt-2*, *wnt-5a*, *wnt-7b* and *wnt-10b* are expressed in young virgin mice during ductal development (5-6 weeks of age) and also during early pregnancy. Additionally, during the whole duration of pregnancy, expression of *wnt-4*, *wnt-5b* and *wnt-6* is observed, suggesting an involvement of these family members in lobular development[127]. For one of these genes, *wnt-4*, direct proof for its role in the progesterone-dependent side-branching of ducts during early pregnancy has been obtained by transplanting mammary epithelia from *wnt-4*$^{-/-}$ mice in wild-type recipients[128]. During lactation, expression of *wnt* genes is down-regulated to undetectable levels with exception of *wnt-6*, whereas re-expression of *Wnt-2*, *wnt-5a*, *wnt-5b* and *wnt-7b* has been observed in the mammary gland at day 7 of involution. However, because no expression data for early involution time-points or for the induction phase of involution are available, we do not know which Wnt(s) potentially interact with SFRP4 at the onset of mammary gland involution. We can also not name the one or more Frizzled receptors that transduce the Wnt signal responsible for maintaining MEC viability. Knowledge of the identity of the ligand-receptor pair(s) found in the mammary gland at the beginning of involution would give some clue on the Wnt signalling branch involved. From the evidence presented in the literature (see 1.2.4), we conclude that the signalling branch related to MEC survival is the canonical Wnt/β-catenin pathway; however, this conclusion could be reflecting the fact that much more is known about canonical signalling than about the other, noncanonical pathways. Advances in the field of noncanonical Wnt signalling will tell us if factors other than those involved in the Wnt/β-catenin pathway have to be considered. Concerning the cross-talk

between the Wnt signalling with other pathways, based on literature data, it is possible to postulate a multitude of interactions occurring in MEC that affect their survival. However, to detect such potential links and to assess their relevance in the context of SFRP4-mediated Wnt signalling inhibition we need a model allowing us to collect experimental and not only correlative evidence.

1.3.2 Objectives of the PhD thesis

Because cells in culture can readily be manipulated and fulfil the requirements of an experimental model, we decided to first set up a cell culture system allowing us to modulate the pathway activity using Wnt(s), SFRP4 and other antagonists and then to examine the effect of these treatments with respect to other signalling pathways. It has to be noted that apoptosis will not be monitored in this approach, since cell lines are notoriously resistant to physiological signals leading to apoptosis. For the establishment of a suitable cell culture model, the following requirements have to be met: 1) the preparation of biologically active Wnt protein for stimulation of cells, 2) the development of assays for assessing the pathway activity at different levels of signal transduction, 3) the characterisation of the response of recipient cells to Wnt stimulation and 4) the preparation of secreted antagonists for use in signalling inhibition experiments. The results obtained while setting up this cell culture model are presented and discussed in section 3; here I want to mention that some choices were dictated by the availability of reagents or by the feasibility of the experiments, rather than by the biological relevance. For example, the use of Wnt-3a for stimulation of cells in our model is motivated by its availability, even though it has a transforming effect in MEC and is not naturally expressed in the mammary gland. Furthermore, it has to be said, that several failed attempts to establish particular features of our model (e.g. cell stimulation with Wnt-4) will not be discussed unless relevant for future work.

2. Material and methods

2.1 Cell cultures and cell fractionation

NIH-3T3, C57MG, and L cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and with insulin (5 µg/ml) and EGF (10 ng/ml) in the case of C57MG cells. The Wnt-3a-producing L cells were purchased from ATCC (CRL-2647) and cultured as the other cell lines. C57MG cells with tetracycline-repressible Wnt-1 gene expression (cell line 2-69-23[129], a gift from H. Clevers) were supplemented with 500 ng/ml tetracycline for repression of Wnt-1 expression. For generation of cytoplasmic fractions, confluent cells from a 5 cm plate were incubated for 10 min in 500 µl of hypotonic lysis buffer (see Appendix B) on ice. Following Dounce homogenisation, samples were centrifuged at 20'000 *g* for 15 min and the collected supernatant represented the cytoplasmic fraction. After protein concentration determination using the Bradford-based Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA), the cytoplasmic lysates were diluted to 1X SDS-PAGE sample buffer (80 mM Tris (pH 6.8), 2% SDS, 10% Glycerol, 2% 2-mercaptoethanol and 0.02% bromphenol blue) and boiled for 5 min. Total cell lysates were collected by lysing cells directly with 1X SDS-PAGE sample buffer (500 µl for a 5 cm cell culture dish) and boiling the lysates for 5 min.

2.2 SDS-PAGE and silver staining

Electrophoretic separation of proteins was performed by discontinuous (Laemmli) SDS-PAGE using the Mini-PROTEAN® II electrophoresis cell (Bio-Rad) with an electrophoretic separation length of 7 cm. For immunoblot analysis of Dvl3, 7.5% polyacrylamide gels were used; otherwise 10% polyacrylamide gels were poured using research-grade 29:1-Acrylamide/Bisacrylamide (Serva, Heidelberg, Germany). To ensure that equal amounts of protein were loaded, protein concentration was determined by using a Bradford-based assay (Bio-Rad Protein Assay) and/or estimated by Coomassie staining of gels run in parallel and confirmed by Ponceau S staining of Western blot membranes. For visualisation of proteins by the silver staining method, polyacrylamide gels were soaked in 50% methanol for at least 4h and the washing solution changed at least 8 times. For silver impregnation, the gels were then soaked in a freshly prepared silver nitrate solution (0.75% $AgNO_3$, 20 mM NaOH, 0.3% NH_4OH) for 12 min and then washed with frequent changes of MilliQ-H_2O for 4 min. For

development the gels were soaked in development solution (0.075% formaldehyde, 1 mM $C_6H_8O_7 \cdot H_2O$) for 2 min, then the reaction was stopped by soaking the gels with a 50% Agfa X-ray film fixer solution for another 2 min. After thorough washing with deionised water, the results were documented by gel photography.

2.3 Purification of Wnt-3a

To obtain Wnt-3a conditioned medium, 48h-old L Wnt-3a cells were cultured for 2 days in DMEM with 10% FCS and this medium then harvested, cleared from cellular debris by centrifugation and stored at 4°C until needed. 600 ml of such conditioned medium was adjusted to 1% Triton X-100, filtered through a 0.2-µm Millipore filter and applied to Blue (Cibacron blue F3G-A) Sepharose 6 Fast Flow (Amersham Biosciences, Little Chalfont, England) column (bed volume of 50ml), which was previously equilibrated in binding buffer (150 mM KCl, 20 mM Tris-HCl pH 7.5, 1% Triton X-100). The column was then washed, first with 150 ml of binding buffer and then with 50 ml of binding buffer containing CHAPS instead of Triton X-100. Bound proteins were eluted using 80 ml elution buffer (1.5 M KCl, 20 mM Tris-HCl pH 7.5, 1% CHAPS) and collected in 40 fractions. An aliquot of each fraction was then adjusted to 1X SDS-PAGE sample buffer and proteins were separated by SDS-PAGE and visualised using the silver staining procedure as described above.

For stimulation experiments involving purified Wnt-3a, recipient cells (L or C57MG cells) were grown to confluence for 48h in 5 cm cell culture dishes using 5 ml culture medium. 2 ml of medium was collected, supplemented with purified Wnt-3a (10µl per ml medium) or elution buffer (control) and re-applied to the cells after removing the remaining 3 ml of culture medium. For the dose-response experiment, serial dilutions of purified Wnt-3a were prepared using elution buffer. For the stimulation experiment performed with Wnt-3a-conditioned medium, the medium from 48h-old, confluent recipient cells was replaced with 3 ml of Wnt-3a-conditioned medium (see above), with 3 ml of control conditioned medium (conditioned by L cells) or with 3 ml of serially diluted Wnt-3a-conditioned medium using control conditioned medium as diluent.

2.4 Antibodies, immunoblot analysis and immunoprecipitation

Polyclonal antibodies against β-catenin, Dvl3 and CK2α were raised in rabbits in our laboratory by Ms. Thekla Constantinou. In brief, the antigens were expressed in E. coli using the inducible pGex expression system (Amersham Biosciences) and purified by preparative SDS-PAGE. Rabbits were immunised repeatedly with 500 μg of antigen, first with Freund's complete and then Freund's incomplete adjuvant. For affinity purification, the sera were incubated with antigen coupled to Affigel-10 (Bio-Rad) and specific antibodies were then eluted using 100 mM glycine buffer (pH 2.5) and dialysed against PBS. The antibody against Wnt-3a was a kind gift from R. Nusse and the remaining antibodies were purchased: the mouse monoclonal antibody against cyclin D1 (sc-20044) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), the mouse monoclonal Anti-Active-β-Catenin (αABC) antibody was from Upstate (Lake Placid, NY, USA), the biotinylated anti-mouse IgG were from DakoCytomation (Glostrup, Denmark), the anti-rabbit IgG, the anti-phospho-ser473 PKB and the anti-PKB antibodies were from Cell Signalling Technology (Beverly, MA, USA).

For Western Blot analysis, proteins were separated by SDS-PAGE as described above and transferred onto nitrocellulose membranes (Schleicher & Schuell Inc., Dassel, Germany) using a semidry electroblotter (Bio-Rad). After transfer, the membranes were incubated for 1h in blocking solution (20 mM Tris pH 7.6, 140 mM NaCl, 0.1% Tween-20, 5% w/v non-fat dry milk) and then washed three times for 10 min with Tris-buffered saline containing Tween-20 (TBS-T: 20 mM Tris pH 7.6, 140 mM NaCl, 0.1% Tween-20). Primary antibodies were diluted 1:1000 or 1:500 in the case of anti-cyclin D1 and αABC with TBS-T containing 5% w/v BSA and the membranes were incubated overnight at 4°C with gentle agitation. After three 10 min-washes with TBS-T, the membranes were incubated for 1h at room temperature in blocking buffer containing the secondary antibody diluted 1:1000. When the biotinylated secondary anti-mouse antibody was used, membranes were further incubated with an avidin-biotin complex conjugated to horseradish peroxidase according to the manufacturer's recommendation (DakoCytomation). After washing the membranes three more times for 10 min with TBS-T, the immunoreactive bands were visualised by enhanced chemiluminescence (using SuperSignal® West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA) and by exposing the membranes to a CCD camera system (Lumi-Imager™, Boehringer Mannheim, Germany). For immunoblot analysis of PKB, proteins were separated on a Novex® Tris-Acetate gel using the NuPAGE® system from Invitrogen (Carlsbad, CA) and transferred on a Invitrolon™ PVDF membrane (Invitrogen) following the manufacturer's protocol. After immunodetection of

Ser473-phosphorylated PKB as described above, the membrane was stripped by incubation for 30 min at 50°C with gentle agitation in stripping buffer (62.5 mM Tris-HCl pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS), washed twice for 10 min with TBS-T and reprobed for total PKB.

For immunoprecipitation, cell lysates were prepared by incubating confluent cells for 10 min in 0.5 to 1 ml Triton X-100 lysis buffer (see Appendix B) on ice and then by clearing them with centrifugation. Protein concentration was determined using the Bio-Rad Protein Assay and 100 µg of protein were diluted to a final volume of 300 µl immunoprecipitation buffer (immunoprecipitation buffer consisted of Triton X-100 lysis buffer mixed 1:1 with hypotonic lysis buffer). Protein samples were incubated for a total of 3h rotating at 4°C with 5 µl of anti-Dvl3 or anti-CK2α antibody. After the first 1.5h of incubation time, 20 µl of protein G sepharose beads (Amersham Pharmacia Biotech), which had been previously washed three times with ice-cold PBS and once with immunoprecipitation buffer, were added. The immunoprecipitated complexes were washed three times in immunoprecipitation buffer, boiled for 3 min in 20 µl of 2X SDS-PAGE sample buffer and analysed by Western blot as described. The phosphatase treatment of the Dvl3-immune complexes was performed by washing the protein G sepharose beads a fourth time with 10 mM Tris-HCl (pH 8), resuspending the beads in 50 µl reaction buffer (50 mM Tris-HCl, 0.1 mM EDTA, pH 8.5) and then incubating them for 30 min at 37°C with 3U alkaline phosphatase and for another 30 min at 37°C after addition of 3U of fresh alkaline phosphatase. Subsequently, the beads were pelleted, resuspended in 20 µl of 2X SDS-PAGE sample buffer and boiled for 3 min.

3. Results and Discussion

The data obtained while establishing and validating our cell culture model are presented and discussed in the following sections. In section 3.1, the purification of Wnt-3a protein is presented and evidence for its biological activity is given. In section 3.2, the consequence of treating cells with purified Wnt-3a is documented by monitoring the Wnt-elicited effects at different levels of the canonical pathway and this section is subdivided accordingly. Two cell lines have been used: L cells and C57MG cells. The L cells, which are of embryonic fibroblast origin, have been shown to produce and release exogenous Wnt-3a efficiently[9] and since they are transformed by it, a measurable effect upon treatment with purified Wnt-3a can be anticipated. More relevant for our studies of MEC survival is the use of the mammary epithelial cell line C57MG, a cell line that has been often used in the context of Wnt signalling. In order to characterise the Wnt-3a-elicited response in these two cell lines, the time response of stimulation was determined by treating cells for different periods of time and the outcome is presented in section 3.2. Finally, the question of a potential cross-talk between Wnt and PKB signalling has been addressed, analysing the effect of Wnt-3a stimulation on the phosphorylation status of PKB, and results are shown in the last section (3.3).

3.1 Purification of biologically active Wnt-3a

The finding that Wnt proteins are hydrophobic and post-translationally modified by palmitoylation explains their poor solubility, a property that had hampered most of the earlier attempts (including ours) to isolate Wnts in a biologically active form, and has to be considered when devising a purification procedure for Wnts. Another development essential for successful production and purification of biologically active Wnts, is the use of a protein expression system that meets some recently described conditions[9]: 1) L cells should be used for transfection, because they yield high amounts of secreted proteins, 2) clones of transfected cells expressing the highest amounts of Wnt protein have to be selected and 3) expression vectors coding for non-tagged versions of Wnt proteins should be employed, since insertion of marker epitopes diminishes the activity of the protein. Hence, using Wnt-3a secreting L cells (see section 2.1) and a published purification protocol with some modifications (see ref.[9] and 2.2), we are now able to prepare Wnt-3a in a relatively pure and, most importantly, biologically active form. In order to monitor the chromatographic purification of Wnt-3a on Cibacron Blue

Agarose and to identify the eluted fractions containing Wnt-3a, the proteins eluted from the column in different fractions were analysed by SDS-PAGE and visualised by silver staining (Figure 3.1a, top panel). Wnt-3a is detectable as a discrete band (arrowhead) with intensity above background levels starting from fraction 26. At this point of the elution process and especially in the following fractions, a significant decrease in the amount of contaminating proteins can also be observed. However, appearance of the Wnt-3a band is not only due to decreased background caused by other proteins, but is also caused by a strong increase in the amount of Wnt-3a that elutes starting from fraction 25 and peaking in fractions 28 and 29 (shown by immunoblotting in Figure 3.1a, bottom panel). Further purification of Wnt-containing fractions can be performed by size exclusion chromatography and cation exchange with heparin chromatography, but it is also possible to directly use the fractions containing the purest Wnt-3a, i.e. the fractions with the highest amount of Wnt-3a relative to contaminating proteins. Hence, fractions 30 and 31 were pooled and this preparation of partially purified Wnt-3a (henceforth referred to as "purified Wnt-3a") was used for further experiments. In order to assess if after the purification process Wnt-3a retained its biological activity, L cells were treated for 3 hours with decreasing amounts of purified Wnt-3a or, for comparison, with medium conditioned for 48 hours by L Wnt-3a cells. As a measure for pathway activation, the level of β-catenin in cytoplasmic cell lysates was determined by immunoblot analysis as described (see 3.2.1 for more details). A clear increase in the amount of cytoplasmic β-catenin is visible when comparing cells treated with undiluted Wnt-3a-containing medium (100%) and cells treated with control medium (0%), i.e. medium conditioned by L cells not expressing Wnt-3a (Figure 3.1b, left). However, there was no clear linear correlation between the level of cytoplasmic β-catenin and the amount of Wnt-3a-containing medium used for cell treatment. When Wnt-3a-containing medium diluted 1:2 (50%) or 1:4 (25%) with control medium is used to stimulate cells, there is no clearly visible decrease in cytoplasmic β-catenin levels, whereas treatment with Wnt-3a-containing medium diluted further results in somewhat reduced levels of β-catenin. This observation can partly be explained by unequal loading of the gel (shown by Coomassie staining), but when compared to the clearly linear dose-dependent response elicited when using purified Wnt-3a (Figure 3.1b, right), one might speculate that Wnt-3a-containing medium also contains some other factor that can potentiate the Wnt-3a effect. To note is that by treating L cells with 10 µl of purified Wnt-3a, stronger pathway activation can be observed, as measured by accumulation of β-catenin in the cytoplasm, than when using undiluted Wnt-3a-containing medium. Using less than 2.5 µl of purified Wnt-3a does not result in an increase of

cytoplasmic β-catenin above baseline levels. These data show that purification of Wnt-3a from medium conditioned by L Wnt-3a cells does not lead to an appreciable loss in its activity and suggest that it is of advantage to use purified Wnt-3a instead of conditioned medium, the latter possibly containing factors interfering with the effect of Wnt-3a.

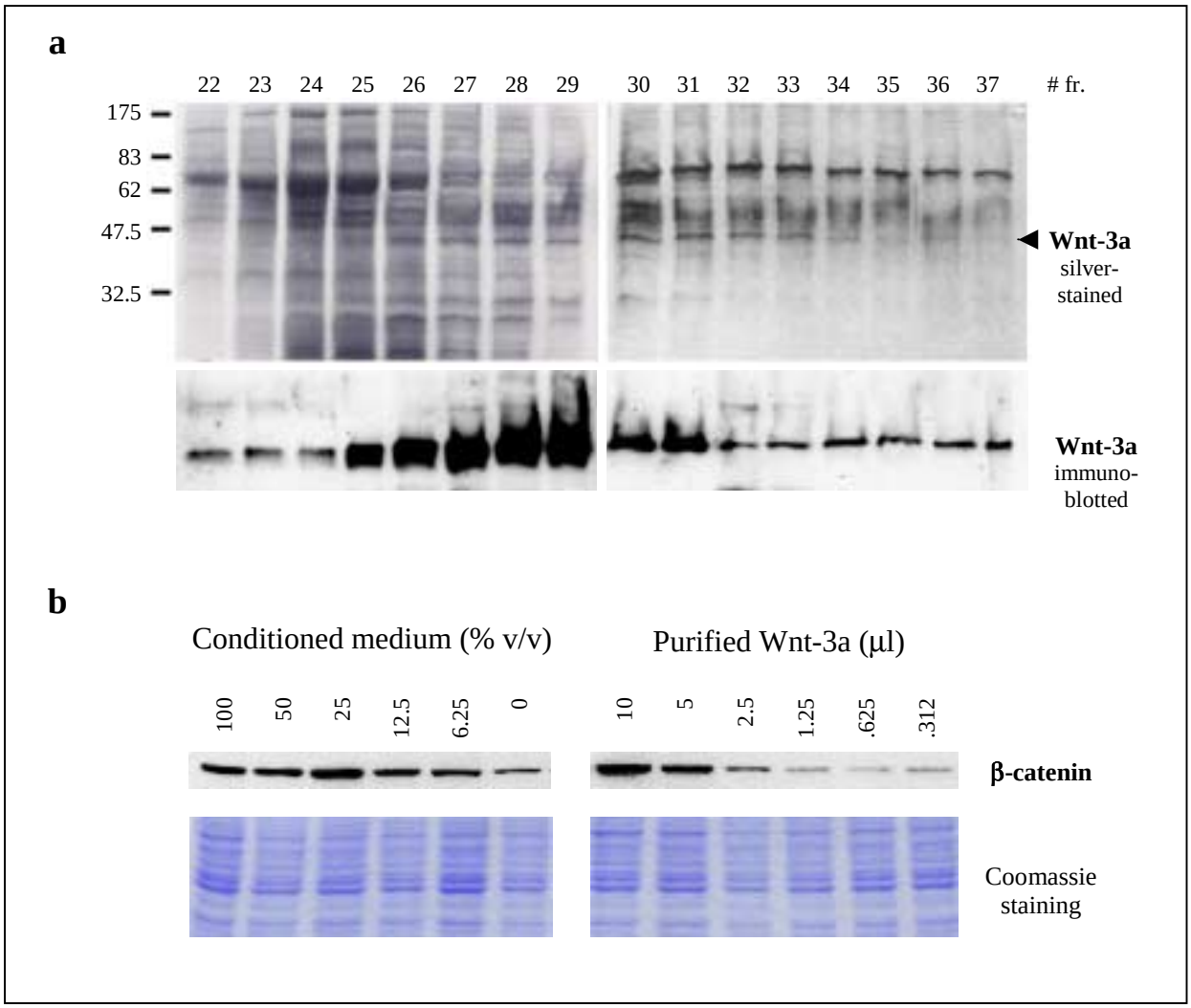

Figure 3.1 Purification of biologically active Wnt3a. **a** Silver-stained SDS polyacrylamide gels containing fractions obtained during Cibacron Blue Agarose chromatography reveals the enrichment of Wnt-3a (top panel) and is confirmed by immunodetection of Wnt-3a (bottom panel). Lanes are labelled with the number of the loaded fraction (# fr.). Size markers are in kilodaltons. **b** Immunodetection of β-catenin in cytoplasmic cell lysates demonstrates activation of the Wnt pathway upon treatment of L cells with different amounts of either Wnt-3a conditioned medium (left) or purified Wnt-3a (right). As a loading control Coomassie staining was used.

3.2 Canonical Wnt pathway activation by purified Wnt-3a

We decided to monitor activation of the Wnt pathway at different levels of signal transduction using assays described in the literature. Due to the central role of β-catenin in the canonical Wnt pathway (see Figure 1.2), analysis of its cytoplasmic accumulation and phosphorylation has been used a measure for pathway activity (3.2.1). As a marker for Wnt signalling at the level of target gene transcription we chose to monitor expression of cyclin D1 (3.2.2). Finally, the Wnt-mediated phosphorylation of Dvl3, representing a pathway component upstream of β-catenin, has also been analysed (3.2.3). We can explain our choice to use these three markers for Wnt pathway activity, by noting that the ability to monitor several pathway components reduces considerably the risk of missing or misinterpret Wnt-mediated signalling effects, especially when considering that several positive and negative regulation mechanisms exist at every level of signal transduction.

3.2.1 Stabilisation of β-catenin

Because β-catenin is the central player in the canonical Wnt pathway, it has been studied intensively and also used very often as a marker for pathway activation. We are using two assays related to the stabilisation of β-catenin to measure Wnt pathway activity: one involves immunodetection of β-catenin that accumulates in the cytoplasm (already shown in Figure 3.1b); the other assay involves immunoblot analysis using a mouse monoclonal antibody specific for the β-catenin dephosphorylated at residues Ser-37 and Thr-41. In Wnt-stimulated cells, β-catenin escapes proteasomal degradation because it is no longer phosphorylated at residues Ser-33, Ser-37, Thr-41 and Ser-45 and as a consequence can not be bound to β-TrCP and the ubiquitin ligase complex. This leads to accumulation of β-catenin in the cytoplasm and translocation to the nucleus (see 1.1.3). Hence, detection of "activated", dephosphorylated β-catenin and of cytoplasmic β-catenin accumulation serves the purpose of monitoring Wnt pathway activity very well. Concerning the assay involving detection of increased levels of β-catenin in the cytoplasm of L and C57MG cells, we found that it is necessary to analyse cytoplasmic cell lysates (rather than total cell lysates) for detection of an increase in the amount of β-catenin in this cell compartment. In fact, when total cell lysates are used, the presence of relatively large amounts of β-catenin from the cell membrane masks the difference in the amount of β-catenin found in the cytoplasm (data not shown). Furthermore, we wanted to

establish whether the ultracentrifugation step could be eliminated from our cell fractionation protocol in favour of convenient centrifugation using a tabletop centrifuge with lower centrifugal force. For this experiment, C57MG cells transfected with a tetracycline-repressible Wnt-1 expression vector were used. Unstimulated (tetracycline-repressed) cells and Wnt-1-stimulated (unrepressed) cells were incubated in hypotonic lysis buffer, broken by Dounce homogenisation (see 2.1) and subjected to a first centrifugation step with a force of 20'000g for 10 minutes. The obtained cleared lysate represents a cytoplasmic fraction which might still contain small cell membrane fragments and organelles, whereas the pellet is composed of the bulk of cell membranes and nuclei. To determine if a significant amount of cell membrane and membrane-bound β-catenin was contained in the lysates cleared after the first low-speed centrifugation, a second centrifugation step using a force of 100'000g was performed. The supernatant was then collected as a membrane-free cytoplasmic lysate, whereas the tube used for ultracentrifugation was carefully rinsed with PBS and the invisible pellet, possibly containing membranous contaminants, was resuspended in 1X SDS-PAGE sample buffer. The fractions obtained were then analysed for the presence of β-catenin by Western Blot and the result is shown in Figure 3.2a. When the expression of Wnt1 is repressed by addition of tetracycline (see 2.1), β-catenin is barely detectable in the cytoplasmic fraction obtained by low-speed tabletop centrifugation ($CF^{20'000g}$) and not detectable in the cytoplasmic fractions centrifuged further with high centrifugal force ($CF^{100'000g}$). In stimulated cells, a significantly higher amount of β-catenin is detected in both cytoplasmic fractions, but again less is present in the fraction subjected to ultracentrifugation, showing that some β-catenin from membranous origin contaminates the cytoplasmic fraction cleared by low-speed centrifugation. We would expect to find this contaminating β-catenin in the pellet obtained after ultracentrifugation ($P^{100'000g}$), but this is only partially the case. However, relevant for this experiment is the finding that a clear difference in the β-catenin levels of stimulated and unstimulated cells is visible using either centrifugation protocol, hence we opted to use tabletop centrifugation, which allows to process samples more efficiently. To note is that a relatively large amount of β-catenin is found in the fractions containing membranes and nuclei obtained after first centrifugation ($P^{20'000g}$) and that only a slight difference in β-catenin levels can be observed between unstimulated and stimulated cells, showing that the membrane-bound pool of β-catenin seems not to be affected by Wnt signalling. To confirm that the observed differences are not due to unequal gel loading, a parallel SDS-PAGE was performed and the gel was stained using Coomassie blue.

In order to characterise the time-dependent response elicited by purified Wnt-3a in the context of β-catenin turn-over, L and C57MG cells were treated for half an hour, one hour, two, four and eight hours by supplementing 2 ml of the cell culture medium with 20 µl of purified Wnt-3a or with 20µl of elution buffer as a control. For each treatment two plates of cells were used, so that cytoplasmic cell lysates and total cell lysates could be prepared in parallel. Both cell lines responded similarly to the treatments: a slight increase in the level of cytoplasmic β-catenin can be observed within half an hour of treatment, but the Wnt-3a effect as detected by cytoplasmic β-catenin accumulation is maximal at two hours and is maintained thereafter for at least 8 hrs (Figure 3.2b). This pattern of stimulation is confirmed in the case of L cells using immunodetection of β-catenin dephosphorylated at Ser37 and Thr41 (Figure 3.2b, bottom panel), even though it has to be noted that the effect elicited by Wnt-3a with respect to an increase of dephosphorylated β-catenin is weaker than with respect to cytoplasmic β-catenin accumulation. It has been suggested that Wnt-mediated dephosphorylation of β-catenin at its N-terminus (at residues Ser-33, Ser-37, Thr-41, Ser-45) is not only necessary to avoid its degradation (see1.1.3), but that it also affects the signalling properties of β-catenin. In other words, accumulation of β-catenin and transduction of Wnt signals are separable events, in that effects on stability of β-catenin can be dissociated from its effect on signalling[130]. According to this view, it is important to determine if accumulation of cytoplasmic β-catenin correlates with an increase in N-terminally dephosphorylated β-catenin upon stimulation with a specific Wnt and in a specific cellular context.

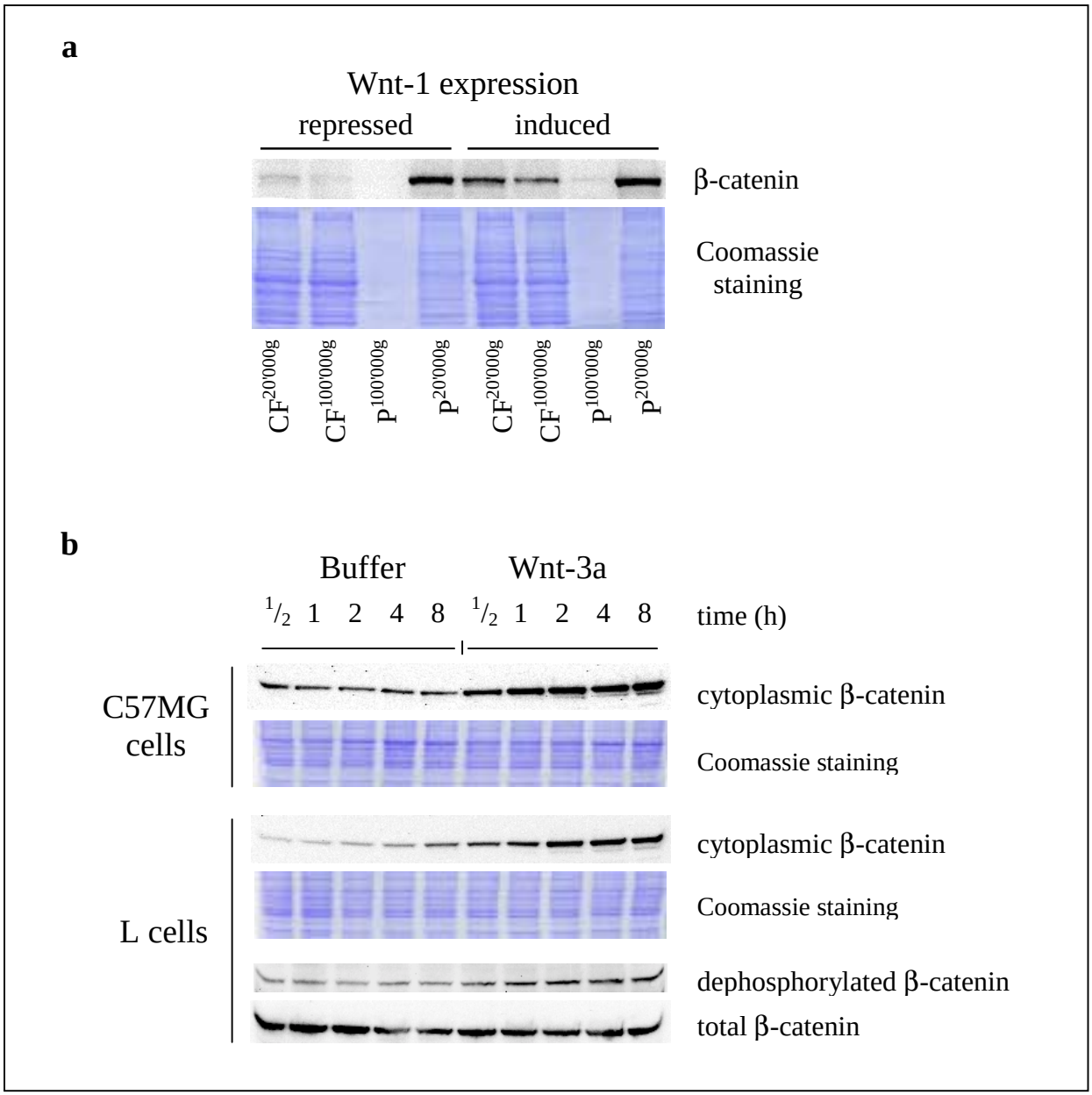

Figure 3.2 Detection of β-catenin stabilisation. **a** Preparation of cytoplasmic cell lysates for β-catenin accumulation assay. C57MG cells stably transfected with an inducible Wnt-1 expression system were caused to swell in hypotonic buffer, broken by Dounce homogenisation and subjected to a first centrifugation step with 20'000g of centrifugal force obtaining a cytoplasmic fraction ($CF^{20'000g}$) and a pellet containing nuclei and membranes ($P^{20'000g}$). Half of the cytoplasmic fraction was subjected to a second centrifugation step using 100'000g to eliminate possible membranous contaminants and obtain a second cytoplasmic fraction ($CF^{100'000g}$) and a second pellet ($P^{100'000g}$). As described in detail in the text, differences in immunodetected β-catenin between unstimulated and stimulated cells can be clearly detected by using low-speed centrifugation, even though some contaminating β-catenin from the cell membrane appears to be present in the cytoplasmic fractions obtained using our cell fractionation protocol. Equal loading is demonstrated by Coomassie staining of a gel run in parallel. **b** Immunodetection of cytoplasmic or dephosphorylated β-catenin. Cells were treated for different spans of time either with purified Wnt-3a or with elution buffer as control and then the elicited effect was

analysed by immunodetection of cytoplasmic β-catenin in C57MG and L cells. An increase in the amount of β-catenin in cytoplasmic lysates can be observed as early as after half an hour of treatment, is maximal at two hours and is maintained thereafter until 8 hrs (Coomassie staining was used to confirm equal loading of the gels). Total lysates of L cells were also analysed for the presence of the activated β-catenin dephosphorylated at Ser37 and Thr41, confirming the kinetics of pathway activation described above (equal gel loading is shown by immunodetection of total β-catenin).

3.2.2 Expression of cyclin D1

To obtain a measure of Wnt pathway activation at the level of target gene transcription, we performed immunoblot analysis of cyclin D1[131]. Because cyclin D1 is not only a target of Wnt signalling, being transcriptionally up-regulated by the β-catenin/TCF complex, but is also regulated in response to other signals, it is important to include adequate controls when using its expression as a marker for Wnt pathway activity. The total lysates of L and C57MG cells obtained in parallel to the cytoplasmic lysates in the time-course experiment of Wnt-3a stimulation were analysed for changes in cyclin D1 transcription and the obtained expression pattern is shown in Figure 3.3. A detectable increase in cyclin D1 levels is observed after stimulation with purified Wnt-3a. However, it has to be noted that the strong increase in the first two time-points is not dependent on Wnt-3a, since also in the control-treated cells a similar amount of cyclin D1 has been detected. This increase probably reflects a stress response due to the handling of the cells and dependent on fos up-regulation. After a short time, this stress-induced effect wears off, and the Wnt-3a induced increase in cyclin D1 levels are visible. Basal levels of cyclin D1 as found in untreated cells are comparable with the level detected in cells control-treated for four hours (data not shown).

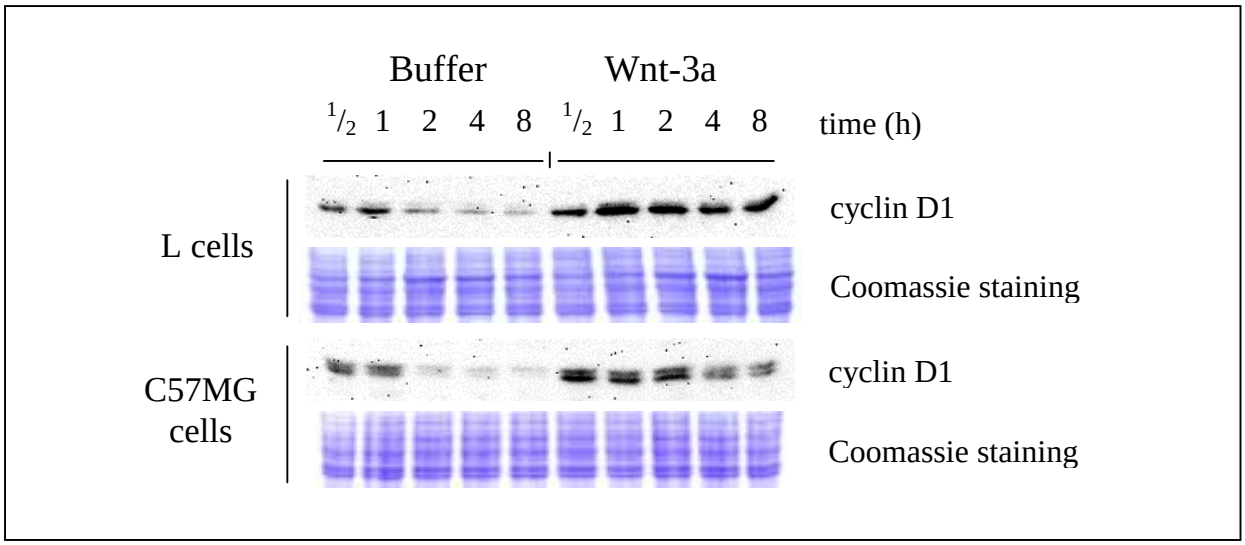

Figure 3.3 Detection of cyclin D1 expression. L and C57MG cells were treated for different times either with buffer or with purified Wnt-3a and then total cell lysates were analysed by immunoblot for changes in levels of cyclin D1. A Wnt-3a-dependent increase in cyclin D1 is detected in the later time- points (starting from two hours of treatment), whereas in earlier time-points a Wnt-3a-independent effect on cyclin D1 expression, probably a fos-dependent stress-induced response due to the handling of the cells, was invariably found. Coomassie stained gels run in parallel serve to confirm equal loading.

3.2.1 Phosphorylation of Dvl3

Dvl has been shown to be hyperphosphorylated upon Wnt stimulation, a post-translational modification that can be visualised as a shift in electrophoretic mobility by immunoblot analysis[132]. Furthermore, because it is known that Dvl is bound and gets phosphorylated by CKII, we have explored the possibility to use co-immunoprecipitation experiments in order to detect an increased amount of CKII-bound Dvl in stimulated cells as a marker for Wnt activity. As shown in Figure 3.4a, Dvl3 is detected by Western Blot as a band doublet with a molecular size of about 90kDa and consisting of hypophosphorylated Dvl3 (lower band) and hyperphosphorylated Dvl3 (upper band, see below). The appearance of the band doublet (presence and position of the bands forming the doublet) varies depending on the treatment and cell type analysed. In unstimulated L and C57MG cells, i.e. cells that were control-treated with elution buffer, similar band doublets are detected (first and third lane of immunoblot shown in 3.4a). When L cells are stimulated for 3 hours with 10 µl of purified Wnt-3a, the lower of the two bands disappears, whereas the upper band becomes somewhat diffuse, especially on the upward facing side (second lane). When C57MG cells are stimulated using the same conditions, the lower band again disappears and the smear observed on the upward facing side of the higher band becomes visible as a new, discrete band (fourth lane).

The interpretation for this result is that Wnt-stimulation results in a somewhat heterogeneous hyperphosphorylation of a part of Dvl3, which then migrates more slowly during electrophoresis as compared to the hypophosphorylated form. To confirm that the differences in electrophoretic mobility are indeed due to differences in phosphorylation status, Dvl3 over-expressed in NIH-3T3 cells was immunoprecipitated and treated with alkaline phosphatase. After dephosphorylation by alkaline phosphatase, immunoprecipitated Dvl3 is detected as a single band that corresponds to the lower band of the band doublet, as can be seen when compared to Dvl3 not subjected to dephosphorylation (Figure 3.4b). It has to be noted that between different electrophoretic runs there is a certain degree of variability in the quality of the electrophoretic resolution between hypo- and the hyperphosphorylated forms of Dvl3, i.e. it is not always possible to detect sharp band doublets consisting of two discrete bands. However, electrophoretic mobility of Dvl3 from cells stimulated with Wnt-3a is always affected, so that it is possible to detect at least a band shift indicating hyperphosphorylation. Due to the relatively large size of Dvl3, resolution is increased by performing SDS-PAGE with gels containing 7.5% polyacrylamide, but other parameters, that in our hands can not be controlled, affect resolution and hence appearance of Dvl3 in immunoblot analysis. An example for suboptimal detection of hyperphosphorylated Dvl3, is shown in Figure 3.4c, where immunodetection of Dvl3 from control- or Wnt-3a-treated C57MG cells resulted in the detection of a shift in electrophoretic mobility only and not as a clear change in the appearance of the band doublet. However, also in this case a difference between unstimulated and stimulated cells is observable, and the time-dependent effect of Wnt-3a already described above is confirmed, even though no clear pathway activation is visible as early as after half an hour using this assay.

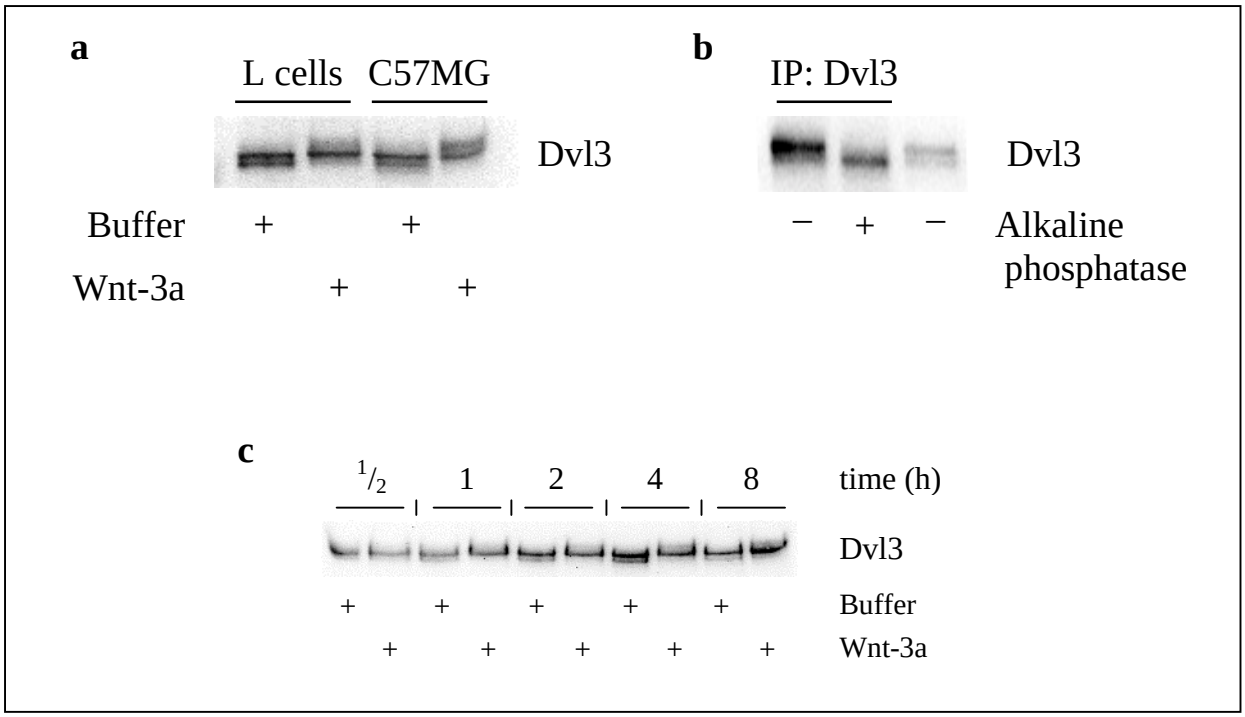

Figure 3.4 Dvl3 as a marker for Wnt pathway activation. **a** Immunoblot analysis of L and C57MG cells using anti-Dvl3 antibody shows that Wnt3a causes a reduction in electrophoretic mobility of a part of Dvl3 resulting in a change of the appearance of the band doublet detected in control-treated cells (Buffer). **b** Alkaline phosphatase treatment of immunoprecipitated Dvl3 (over-expressed in NIH-3T3 cells) results in disappearance of the more slowly migrating, upper band of the band doublet, showing that this band consists of hyperphosphorylated Dvl3. **c** Detection of a time- and treatment-dependent shift in electrophoretic mobility of Dvl3 by immunoblotting.

It has been shown by co-immunoprecipitation experiments that Dvl3 binds CKII[132] and we wanted to confirm this observation using Wnt-3a-stimulated cells. Additionally, we wanted to assess if different amounts of Dvl bind to CKII depending on the activation status of the Wnt pathway, i.e. if regulation of Dvl phosphorylation possibly occurs at the level of substrate binding. Lysates from control-treated (10 µl elution buffer) cells and from cells treated for 3 hours with 10 µl purified Wnt-3a were incubated with anti-CKIIα antibody or anti-Dvl3 antibody and the immunoprecipitated proteins were then analysed for the presence of Dvl3 by Western Blot. In both control-treated and stimulated L cells, a comparable amount of Dvl3 co-precipitates with CKII (first and third lane of immunoblot shown in Figure 3.5a, left panel). This result confirms the association between Dvl3 and CKII, however, we find that the same amount of CKII-bound Dvl3 is present in L cells (and C57MG cells, data not shown) independently of the degree of Wnt pathway activation, implying that Wnt stimulation does not affect the amount of Dvl3 bound to CKII. This result is not so surprising when considering that activation of Wnt signalling also does not affect the amount of CKII itself as shown by

immunodetection of CKII in lysates obtained in a parallel experiment in which pathway activation is confirmed by detection of cytoplasmic β-catenin and cyclin D1 accumulation (Figure 3.5b). Because the amount (band intensity) and phosphorylation (electrophoretic mobility) of CKII-bound Dvl3 does not change in response to Wnt-3a-stimulation, it appears that Wnt activation does not affect the binding capacity of CKII for Dvl3, raising the question about the mechanism leading to detection of hyperphosphorylated Dvl3 in stimulated cells (as seen in the right panel of Figure 3.5a). A possible explanation for this observation is that only in stimulated cells phosphorylated Dvl3 can accumulate, whereas in unstimulated cells Dvl3 is dephosphorylated. This interpretation, however, is in disagreement with a previous report, where stable transfection of C57MG cells with Wnt1 led to an increase in the amount of CKII and in its activity, as demonstrated by an *in vitro* kinase assay[25]. It will be necessary to measure the activity of CKII so as to confirm the hypothesis that the appearance of hyperphosphorylated Dvl3 in our Wnt-3a-stimulated cells is not due to increased CKII activity, but rather to reduced dephosphorylation by a yet to be identified phosphatase. It has to be mentioned that another kinase, CKI, which is unrelated to CKII and of which several isoforms are known, has also been shown to interact with Dvl and to be implicated in its regulation, further complicating the investigation of the mechanism responsible for Dvl regulation by phosphorylation[133].

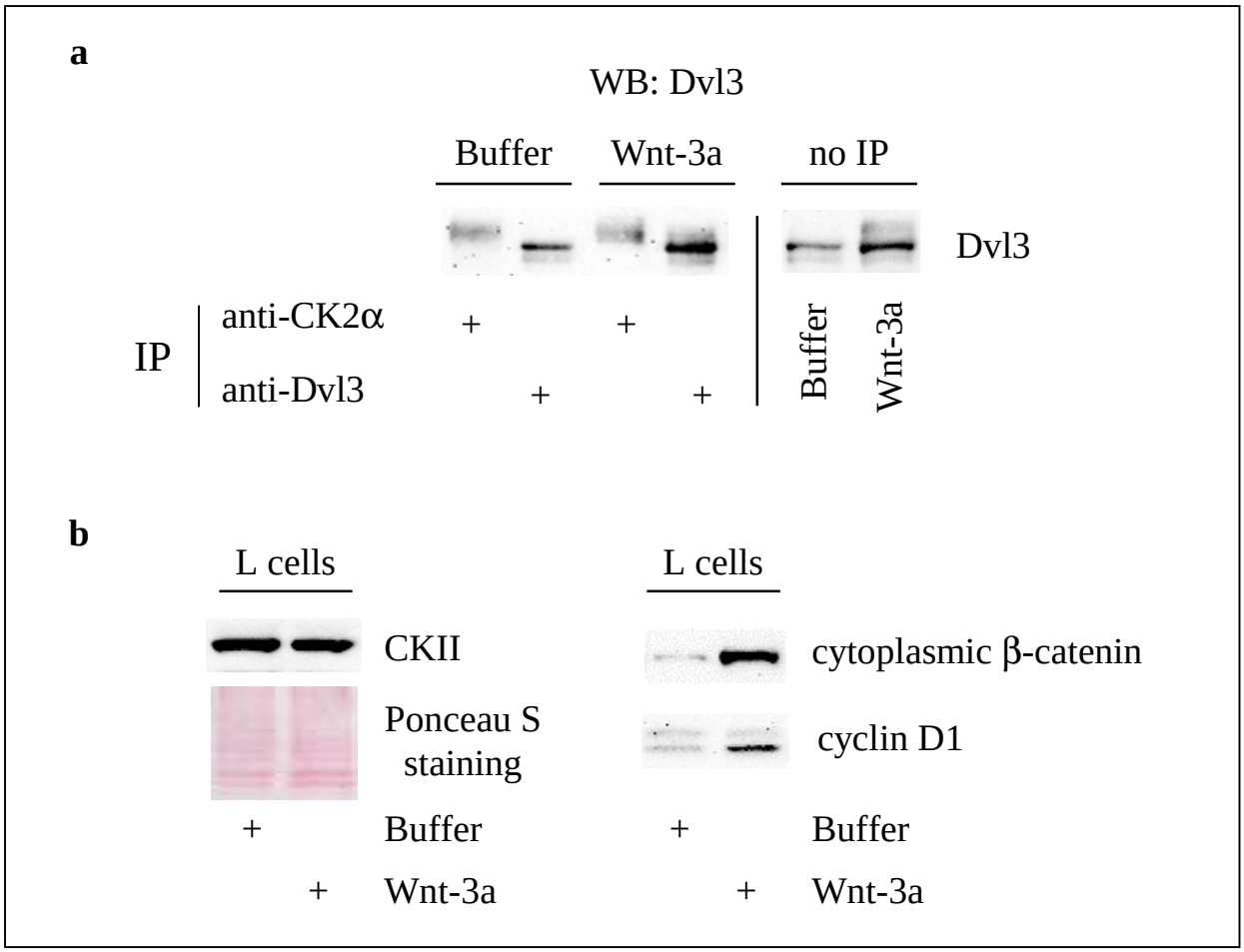

Figure 3.5 Interaction between Dvl3 and CKII. **a** Co-immunoprecipitation of Dvl3 with CKII in cells stimulated with Wnt-3a demonstrates binding of Dvl3 to CKII, a characteristic that is distinctive for both unstimulated and stimulated cells and can therefore not be used as an indicator for Wnt pathway activation. **b** Immunodetection of CKII in lysates from control-treated and Wnt-3a-treated L cells reveals no change in the amount of CKII (equal loading is shown by PonceauS staining of the membrane), whereas the increase of cytoplasmic β-catenin and cyclin D1 confirms that activation of the Wnt pathway has occurred in stimulated cells.

3.3 PKB phosphorylation in Wnt-3a-stimulated cells

The total lysates of L and C57MG cells treated for four hours during the time-course experiment presented above were analysed using an antibody specific for PKB phosphorylation at the Ser473 residue as a measure of PKB activation (Figure 3.6). Stimulation of both L and C57MG cells with Wnt-3a correlates with increased phosphorylation of PKB, confirming previous reports that link Wnt signalling to PKB. To note is the large difference in total amounts of PKB between the two cell lines, which is not due to loading differences as demonstrated by Ponceau S staining (after phospho-specific immunodetection of PKB, the

membrane was stripped and reprobed for total PKB immunodetection and finally stained using Ponceau S). This effect of Wnt signalling on the activity of PKB has been reported before but is controversial (see 1.2.4); our result confirms the occurrence of the postulated cross-talk between Wnt and PKB signalling, but it remains to be determined at which level such an interaction takes place. Independently of the exact mechanism involved, the observed activation of PKB by a Wnt-controlled mechanism is relevant for our hypothesis that Wnt signalling maintains MEC viability by controlling PKB and its important role as survival factor. Furthermore, this causal relationship between Wnt pathway activation and phosphorylation of PKB is consistent with the decreased levels of phosphorylated PKB found in the mammary gland of transgenic mice over-expressing SFRP4, because in this transgenic model over-expression of SFRP4 is postulated to inhibit canonical Wnt signalling and results in increased levels of apoptosis[70].

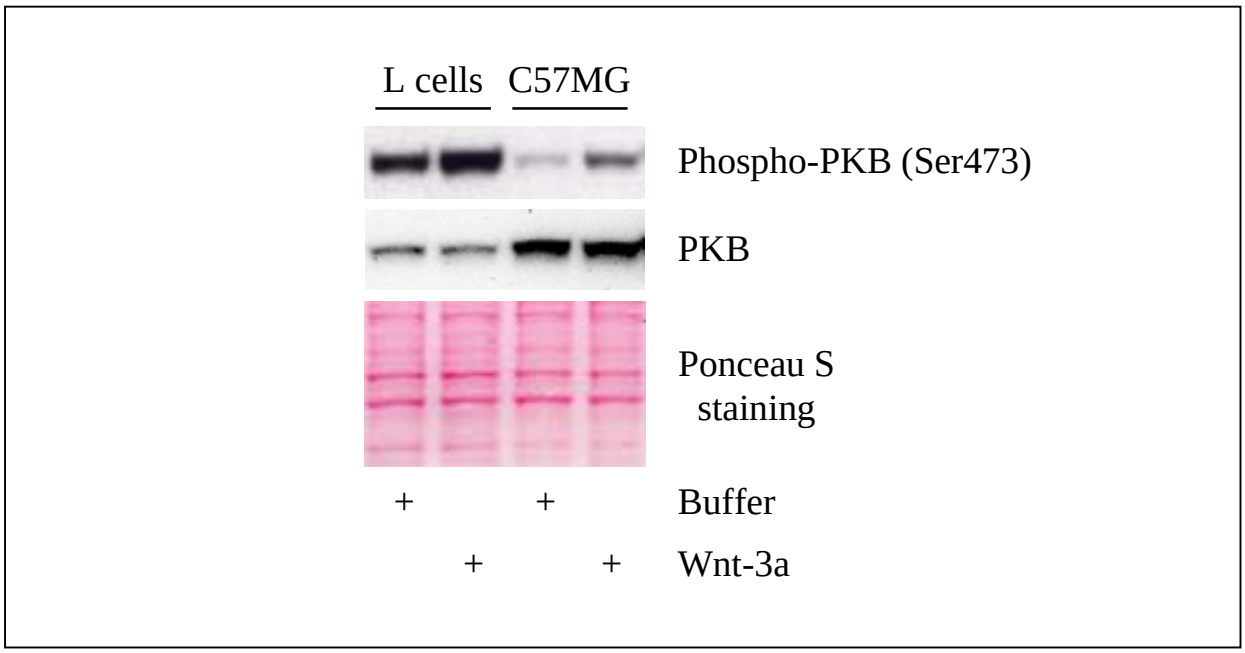

Figure 3.6 Immunoblot analysis of PKB phosphorylation status. Upon stimulation of both L and C57MG cells with purified Wnt-3a for four hours, an increase in phosphorylation of PKB at serine 473 can be observed as compared to control treated cells (top panel). While the same amount of protein was loaded (as shown in the bottom panel by Ponceau S staining of the membrane), C57MG cells show a higher level of total PKB than L cells (middle panel). To note is that there is no difference in the amount of total PKB between control treated and Wnt-3a stimulated cells, so that the observed increase in PKB phosphorylation represents a Wnt-3a-elicited effect.

4. Concluding remarks

The presented results show that using our cell culture system we can activate Wnt signalling in the two different cell lines using purified Wnt-3a and monitor its effect along the signal transduction process from the membrane to the nucleus. However, we developed the cell culture model for testing the ability of secreted Frizzled-related protein 4 to modulate Wnt signalling, but so far every attempt to obtain an effect has failed. We have used three antagonists, the LRP-binding Dkk1 and the two Wnt-binding SFRP4 and SFRP1. These constructs were either introduced as expression vectors directly in the recipient cells by stable transfection or were produced by stably transfected HEK293 cells and the culture medium conditioned by these cells was transferred to recipient cells. None of these approaches has allowed us to modulate the stimulation effect of Wnt-3a. In the case of Dkk1 this result is surprising, since it inhibits signalling in a ligand-independent way by binding to LRPs and the postulated antagonistic effect on Wnt-3a signalling has been reported[134]. Because we are not able to detect expression of Dkk1 in stably transfected cells neither by Northern Blot, nor by western Blot, it seems probable that the expression vectors we obtained from Dr. Cathrin Brisken (ISREC, Lausanne) and Christof Niers (DKFZ, Heidelberg) are malfunctioning and we plan to perform further tests to determine the problem with Dkk1.

Concerning the negative results obtained in inhibition experiments involving SFRP4 and SFRP1, one possible explanation is that they do not interact with Wnt-3a and hence can not inhibit it. Different affinities of SFRPs for Wnts can be expected and have been shown in the case of FRZB, which inhibits the effect of Wnt-1 and Wnt-8 in *Xenopus* embryos, but not of Wnts -3a, -5a or -11[56]. However, because we lack a positive control for pathway inhibition (Wnt ligand-independent Dkk1 inhibition), we can not tell if our inability to regulate Wnt signalling by SFRPs is due to a biological quality of the SFRPs studied in our system or if it is caused by some problems of technical nature.

The information available in the literature about SFRP4 relates mostly to its expression patterns in different model systems, but it is not clear which Wnts interact with SFRP4 or if and how SFRP4 exerts its postulated antagonistic effect. Recent investigations, however, offer experimental evidence for an antagonistic role of SFRP4 in canonical Wnt/β-catenin pathway. In one study using an *in vivo* rat model, infusion of purified recombinant SFRP4 was shown to inhibit renal sodium-dependent phosphate transport and to increase the amount of phosphorylated β-catenin in kidney homogenates[58]. In the second study showing an antagonistic effect of SFRP4 on Wnt signalling, an *in vitro* model has been used involving the human

prostatic carcinoma cell line PC3. Overexpression of SFRP4 in these cells results in a decrease of proliferation and in the inactivation of GSK3β as shown by immunodetection using an antibody specific for GSK3β phosphorylated at the serine 9 residue[59]. Finally, one more study offers experimental evidence for the antagonistic effect of SFRPs and links their function to tumourigenesis by showing that the expression of several SFRP is lost in colorectal tumours due to promoter hypermethylation and that this epigenetic SFRP inactivation might predispose to the development of colorectal cancer by complementing downstream mutations in the Wnt pathway. However, SFRP4 had no or only minimal inhibitory activity as compared to the other SFRPs analysed in this investigation (SFRP1, SFRP2 and SFRP5). Experiments were performed using different colorectal cancer cell lines and several assays were performed to monitor the effect of SFRPs on endogenous Wnt signalling activity as well as on pathway activation by exogenous Wnt1 expression. Especially relevant for our research is the finding that cells transiently transfected with SFRPs showed elevated levels of apoptosis, but again, this was only the case when SFRP1, SFRP2 and SFRP5 but not SFRP4 were used[60]. In summary, these recent studies supply first experimental evidence for an antagonistic effect of SFRP4 and more importantly show, predictably, that this effect depends on the cell-type and experimental context. Hence, even though the potential for a Wnt antagonistic effect of SFRP4 is given, the challenge concerning future studies using the cell culture system developed here will be to find the conditions necessary for observing an effect exerted by SFRP4 on Wnt signalling. Only then it will be possible to address the questions relating to the role of SFRP4 in regulating survival and death of mammary epithelial cells.

Appendix A - Abbreviations

AP-1	Activator protein-1
APC	Adenomatous polyposis coli
BAD	Bcl-2 associated death agonist
Bcl-2	B-cell leukaemia 2 protein
β-TrCP	β-transducin repeat-containing protein
CamKII	Ca^{2+}/calmodulin-sensitive kinase II
CK	Casein kinase
CRD	Cysteine-rich domain
CREB	Cyclic AMP response element binding protein
DAG	Diacylglycerol
Dkk	Dickkopf
Dvl	Dishevelled
ECM	Extracellular matrix
EGF	Epidermal growth factor
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FKHR	Forkhead in rhabdomyosarcoma
Fos	Finkel and osteogenic Sarcoma
Fz	Frizzled
GBP	GSK3β binding protein
Gro	Groucho
GSK3β	Glycogen synthase kinase 3β
IGF	Insulin-like growth factors
IGFBP	IGF binding proteins
IKK	IkB kinases
ILK	Integrin-linked kinase
IP_3	Inositol 1,4,5-trisphosphate
IκB	Inhibitor of NF-κB
Kny	Knypek
Krm	Kremen
LDL	Low density lipoprotein

LRP	LDL-receptor-related protein
MAPK	Mitogen-activated protein kinase
MEC	Mammary epithelial cell
MMTV-LTR	Mouse mammary tumour virus long terminal repeat
NF-κB	Nuclear factor kappa B
$p90^{RSK}$	90kDa ribosomal S6 kinase
PCP	Planar cell polarity
PDGF	Platelet-derived growth factor
PDK1	3-phosphoinositide dependent protein kinase 1
PDZ	Post Synaptic Density-95, Discs-large and Zonula occludens-1
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5 biphosphate
PIP_3	Phosphatidylinositol 3,4,5 triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PP2A	Protein phosphatase 2A
PRLR	Prolactin receptor
RTK	Receptor tyrosine kinase
SFRP	Secreted Frizzled-related protein
Src	Rous sarcoma oncogene cellular homolog
Stat	Signal transducer and activator of transcription
TCF	T-cell factor
TEB	Terminal end bud
WAP	Whey acidic protein
WIF-1	Wnt inhibitory factor 1
WISP-1	Wnt1-induced secreted protein

Appendix B - Cell lysis buffers

Hypotonic lysis buffer

1X low-salt phosphate buffer (see below)
3 mM MgCl
10% sucrose (cell culture grade)
1 mM 2-mercaptoethanol

Protease inhibitors: 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM β-glycerophosphate, 1 mM 6-aminohexanoic acid, 1 mM NaF, 1 mM Na-orthovanadate, 1 mM PMSF (added just prior to use)

Low-salt phosphate buffer (4X): 16 ml of a 0.2 M NaH_2PO_4 solution, 84 ml of a 0.2 M $KHPO_4$ solution and 100 ml MilliQ-H_2O (the pH was adjusted to 7.5)

Triton X-100 lysis buffer

1X PBS (see below)
1% Triton X-100
1 mM EDTA
1 mM EGTA

Protease inhibitors: 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM β-glycerophosphate, 1 mM 6-aminohexanoic acid, 1 mM NaF, 1 mM Na-orthovanadate, 1 mM PMSF (added just prior to use)

PBS (10X): 0.1 M Na_2HPO_4, 17.6 mM KH_2PO_4, 1.37 M NaCl, 26.9 mM KCl (the pH was adjusted to 7.4 with HCl)

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