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Review

Complexity of the Wnt/β-catenin pathway: Searching for an activation model

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ABSTRACT

Wnt signaling refers to a conserved signaling pathway, widely studied due to its roles in cellular communication, cell fate decisions, development and cancer. However, the exact mechanism underlying inhibition of the GSK phosphorylation towards β-catenin and activation of the pathway after biding of Wnt ligand to its cognate receptors at the plasma membrane remains unclear. Wnt target genes are widely spread over several animal phyla. They participate in a plethora of functions during the development of an organism, from axial specification, gastrulation and organogenesis all the way to regeneration and repair in adults. Temporal and spatial oncogenetic re-activation of Wnt signaling almost certainly leads to cancer. Wnt signaling components have been extensively studied as possible targets in anti-cancer therapies. In this review we will discuss one of the most intriguing questions in this field, that is how β–catenin, a major component in this pathway, escapes the destruction complex, gets stabilized in the cytosol and it is translocated to the nucleus where it acts as a co-transcription factor. Four major models have evolved during the past 20 years. We dissected each of them along with current views and future perspectives on this pathway. This review will focus on the molecular mechanisms by which Wnt proteins modulate β-catenin cytoplasmic levels and the relevance of this pathway for the development and cancer.

1. Introduction

Wnts are a family of secreted lipid-modified glycoproteins involved with paracrine and autocrine signaling events. They are broadly studied due to their influence on processes such as embryonic development, regeneration, cancer and cellular differentiation [1–5].

The first Wnt gene mapped was found in a *Drosophila melanogaster* recessive mutation screening. It was characterized by segmentation defects resulting in wingless flies [6]. About a decade later, scientists investigating a model of induced mammary carcinoma in mice, driven by DNA integrations of the mouse mammary tumor virus (MMTV), identified a proviral activation of a putative mammary oncogene (*int-1* for integration site 1) on mouse chromosome 15 [7]. Interestingly, analysis of the genetic mapping of the *Int-1* into host DNA and the *Drosophila melanogaster wingless* mutation indicated that *wingless* and *int-1* were in fact orthologues [7,8]. Thus, the term "Wnt" was coined by the junction of the names *Int-1* and *wingless* (Wingless + Int1 = Wnt1) [9].

Over the years, phylogenetic comparisons reviewed that Wnt genes spread across many different phyla of metazoa [10–12]. It is present and functional from simple multicellular organisms such as sponges (Phylum porifera) [13] and the fresh water polyp, *Hydra* (Phylum Cnidaria) [10], through to worms like planaria (Phylum Platyhelminthes) [14], insects (Phylum Arthropoda) [1,6], vertebrates, e.g. the amphibian *X. leavis* [2,15], and mammals including *Homo sapiens* [1,16].

Vertebrates have an elaborated set of Wnt genes and genes associated with Wnt signaling. Together, they are involved in several biological functions, mentioned above [1,3,16,17]. So far, 19 Wnt ligand genes have been identified in humans and mice [1,3,18]. This pathway,

Abbreviations: APC, adenomatous polyposis coli; BCL-9, B-cell lymphoma 9; BMPs, bone morphogenetic proteins; CARM, coactivator-associated arginine methyltransferase; CBP, CREB-binding protein; CK1α, casein kinase 1α; CRD, Cysteine-Rich Domain; DIX, Dishevelled interaction with axin; DKK, Dickkopf; Dvl, Dishevelled; EGF, epidermal growth factor; EVR2, Exudative Vitreoretinopathy 2 protein; GPCR, G-protein coupled receptor; GSK3-β, glycogen synthase kinase 3 beta; HDAC, histone deacetylases; LDLR, low-density lipoprotein receptor; LGR, Leucine-Rich Repeat Containing G-protein coupled receptors; LRP, low-density lipoprotein receptor-related protein; MMTV, mouse mammary tumor virus; NDP, Norrie Disease Protein; SET1, SET Domain Containing 1; sFRP, secreted Frizzled related protein; TCF/LEF, T-cell factor/lymphoid enhancer factor; TLE, transducing-like enhancer of split; β-TrCP, β-transducin repeat-containing protein; WIF1, Wnt inhibitory factor; Wnt, Int-1 + wingless; WTX, Wilms' tumor protein; WT1, Wilm's tumor protein 1

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however, is far more complex than suggested by the number of ligands alone. In the literature, it has been separated into 3 groups: i) the so-called canonical Wnts or β–catenin-dependent (*Wnt2*, *Wnt2b*, *Wnt3*, *Wnt3a*, *Wnt8*) [1,3], ii) β–catenin-independent [19,20], and iii) the non-canonical Wnt (*Wnt5a*, *Wnt11*, *Wnt7a*). Though some Wnts (e.g. *Wnt11*) do not fit this classification, because it can activate both canonical and non-canonical pathways [21,22].

The main focus of this review will be the activation mechanisms of the so-called canonical Wnt signaling pathway, recently mentioned as Wnt/LRP6 β-catenin-mediated transcription pathway [23]. Nevertheless, β-catenin-independent Wnt pathways should not be seem as totally separate since they may cross-talk by sharing components in several cellular processes [24–26].

Over the years, several disease-causing Wnt mutations have been extensively characterized [27–29]. This information is recorded in "The Wnt homepage" (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/human_genetic_diseases), therefore it will not be discussed in this review.

Here we will present the main players of the Wnt pathway and describe four proposed models of activation of the canonical Wnt signaling: i) a classical biochemical model, in which the destruction complex is partially disrupted and retained in close proximity to the plasma membrane in its inactive form; ii) a cell biology model that describes formation of a multivesicular body that traps the destruction complex inside a double layered structure rendering it incapable of phosphorylate β-catenin thus, incapable to tag it to the proteasome-mediated proteolysis pathway; iii) a model in which phosphorylated β-catenin clogs the destruction complex, which in turns, is captured to the plasma membrane and kept in its inactive form; iv) a recently proposed model, which shows the auto-inhibition of the destruction complex driven by Axin conformational changes. All four models describe distinctive mechanisms resulting accumulation of β-catenin in the cytosol, therefore activating the canonical Wnt pathway.

2. WNT ligands and WNT receptors

Two major classes of receptors have substantial roles in activating Wnt signaling. The primary Wnt receptors are called Frizzled receptors, and belong to a very specific G-protein coupled receptor (GPCR) subfamily [30] (Fig. 1A).

2.1. Frizzled receptors

Frizzled receptors have 7 transmembrane-spanning domains, a large N-terminal domain that contains both a signal sequence directing the protein to the plasma membrane, and a Cysteine-Rich Domain (CRD), named after a conserved pattern of 10 cysteine residues found in this part of the mature protein. A large C-terminal domain, that mediates interaction with intracellular proteins, such as Dishevelled (Dvl), G-proteins, Arrestins and a number of intracellular loops that contain phosphorylation sites targeted by intracellular kinases [30–33].

Phylogenetically, there is variation in the number of *Frizzled* genes; for instance, mammals have 10 different genes encoding Frizzled receptors, whereas *D. melanogaster* and *X. laevis* have 4 and 11 orthologues, respectively [30,34]. The name Frizzled was derived from a recessive mutation in *D. melanogaster*, which gives rise to flies with curly and disorganized bristles and cuticle hair, therefore looking frizzled. Frizzled receptors range in length from 500 to 700 amino acids with molecular weight of ~71 kDa. But differences in sequence and post-translational modifications lead to variation on SDS-PAGE separation and in molecular weight determination [34–37].

2.2. The low-density lipoprotein receptor-related protein (LRP)

Wnts have a co-receptor that is required for activation of the β-catenin-dependent Wnt pathway (Fig. 1A). The low-density lipoprotein receptor-related protein (LRP) [35] comprises 12 genes in the mammalian genome, however, LRP5 and LRP6 seems to have a stronger and undeniable connection with the canonical Wnt signaling [1,32,38,39], as their orthologue *arrow* in *D. melanogaster* [40,41]. A role for LRP/arrow in Wnt signaling came from genetic studies showing that mutations in arrow phenocopied the *wingless* phenotype in flies [41]. These observations were the basis for classifying *arrow* as an orthologue of LRP5/6 and for its epistatic position in the Wnt pathway [40,41].

Investigations involving knockout of either or both LRP5/6 in mice confirmed the LRP requirement for activation of the Wnt pathway [42]. A number of other studies using overexpression, deletion and domain-specific deletion of LRPs helped to elucidate their function in the canonical Wnt signaling [43].

LRPs are type I single-pass transmembrane proteins, with a long extracellular N-terminus that mediates interaction with extracellular ligands and/or antagonists of canonical Wnt signaling, and a short C-terminus that mediates interactions with intracellular proteins. The C-termini of LRPs also contain target motifs for kinase phosphorylation thought to be important for Wnt signaling [42–44]. Arrow/LRP5/6 has a small variation in length containing 1678, 1615 and 1613 amino acid residues, respectively, with calculated molecular weights of ~200 kDa. Their extracellular region contains 4 epidermal growth factor (EGF) like domains and 3 low-density lipoprotein-related receptor (LDLR) repeats required for ligand binding [35,41]. The intracellular portion contains the proline-rich PPPSP motif, which is the sequence target of glycogen synthase kinase 3 beta (GSK3-β) phosphorylation required for activation of the β-catenin-dependent Wnt pathway [32,38,43].

3. Extracellular co-activators of WNT signaling

3.1. Norrin or Norrie Disease Protein (NDP) or X-linked Exudative Vitreoretinopathy 2 protein (EVR2)

Norrin or Norrie Disease Protein (NDP) or X-linked Exudative Vitreoretinopathy 2 protein (EVR2) is a cysteine-knot like growth factor protein in humans encoded by the NDP gene [45–47]. Norrin is a extracellular protein that binds and activates Wnt receptors at the plasma membrane [48,49]. Similarities of phenotypes between *frizzled4* mouse mutants, NDPs and mouse model for Norrie disease suggested that Norrin protein might activate Wnt signaling [50–52]. Indeed, Norrin binds to Frizzled 4 on its CRD in the extracellular environment, a process that seems to require Wnt co-receptors LRP5/6 [51,53]. Interestingly, Norrin binds, with high affinity, to the CRD of Frizzled 4, but not to Frizzled 8 [50,52], revealing some degree of specificity to the system. In *Xenopus* embryos, maternal Norrin is necessary to activate Wnt signaling and for the formation of the anterior central nervous system [54]. Norrin can also bind to Lgr4/5 and 6, but only binding to Lgr4 activates Wnt signaling [55]. The complete relationship of Norrin protein and Wnt signaling is not fully understood, but structural analysis had shed light on the binding mechanism and shows how mutation can affect the function of the mature protein [49,50] (Fig. 1A).

3.2. R-spondins

In addition to Wnt ligands, other Non-Wnt molecules are capable of boosting activation of Wnt signaling [56,57]. R-Spondins are a family of secreted proteins that prevent LRP5/6 internalization and potentially enhancing the activation of the canonical Wnt pathway [57]. One early piece of evidence suggesting a link between R-spondin and the Wnt signaling pathway came from an analysis of Wnt-1/3a double knockout mouse that showed less expression of R-spondin on the roof plate during the development of the neural tube, indicating some involvement in dorsal neural tube formation/patterning regulated by Wnts [58]. R-spondins actively participate in the development of vertebrates, functioning extracellularly to regulate receptor-ligand interactions, and synergizing with Wnt ligands during activation of Wnt signaling