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Knockout mouse models to study Wnt signal transduction

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Wnt signal transduction is crucial for maintaining the balance between proliferation and differentiation throughout embryogenesis and postnatal life. Here, we provide a comprehensive overview of the conventional knockout mouse studies of both the canonical and the noncanonical Wnt pathways during mammalian development. Many of these knockout mice display early embryonic lethality, underscoring the fundamental importance of Wnt signal transduction, but precluding functional analyses at later stages. Use of conditional or inducible mouse models will enable us to study the role of Wnt signaling during later stages of development and adult life. Furthermore, genomic-scale approaches and advanced imaging techniques could provide a means to start dissecting the mechanism behind the observed phenotypes.

Studying Wnt signal transduction in mice

The development of multicellular organisms requires the orchestrated activities of various signal transduction pathways to ensure control over processes governing cell proliferation, differentiation and survival. One of the signaling pathways crucial for embryonic development and for maintaining homeostatic tissue function is the Wnt pathway [1–3] (Box 1). Since the 1980s our knowledge of Wnt signal transduction has expanded tremendously. Detailed, current information on this pathway can be found on the Wnt Homepage (http://www.stanford.edu/~rnusse/wntwindow.html).

As it turns out, the Wnt pathway shows evolutionary conservation across a wide range of species, ranging from the freshwater polyp *Hydra* to worms, flies and vertebrates (Box 2). The advent of genetic manipulation of the mouse germ line, enabling the generation of transgenic and knockout mice, has greatly facilitated the analysis of the physiological function of various Wnt pathway components and has proved to be a tremendous resource for scientists interested in resolving the role of Wnt signal transduction during mammalian development, normal physiology and disease. Moreover, studies using transgenic and knockout mice have enabled us to take observations previously made in lower model organisms to the next level, so that we can evaluate whether characteristic findings made in invertebrates, amphibians and mammals represent generic features of Wnt pathway activity or species-specific phenomena.

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In 1990, the first mice designed to carry a null mutation in a Wnt signaling component were *Wnt1*-deficient mice [4,5]. These, like all other first-generation knockouts, were 'conventional' knockout mice, generated by gene targeting through homologous recombination in embryonic stem cells resulting in germline loss of the gene under study. Consequently, all knockout progeny carry the null allele in all cells of the body from embryonic development onwards. This approach has several well-known disadvantages, including the fact that only the first crucial function of the gene under study can be characterized. As such, early embryonic lethality precludes analyses of a role during later stages of development.

Since then, mouse engineering technology has advanced significantly [6] (Figure 1). The advent of *Cre-lox* technology and the availability of additional systems providing regulatable gene expression have resulted in the capability to switch genes off and on at the wish of investigators, enabling both spatial and temporal control over gene expression. A comparable development has occurred in the generation of transgenic mouse lines, which have historically been obtained following injection of the transgene into oocytes. Although transgene expression was usually driven by a tissue-specific promoter, insertion of the transgene into the genome was random, with no control over expression levels. Because these are greatly dependent on both insertion site and copy number, due to concatemerization of the transgenic construct, conditional transgenes, activated only in the tissue and at the time of interest, have enabled more focused studies. Although studies using transgenic mouse strains have yielded a wealth of information regarding the role of Wnt signaling during mammalian development and normal physiology, we will focus here on conventional mouse knockout studies, which were aimed at learning more about the endogenous role of Wnt signal transduction components. We discuss the key findings that have been made upon analyzing these mice and point out the obstacles that are encountered with respect to interpreting some of the phenotypes. Finally, we address how the use of more advanced techniques in future studies could further enhance our understanding of the Wnt signal transduction pathway.

Targeting ligands and receptors

The mouse genome harbors 19 *Wnt*-encoded ligands, ten *Fz* (Frizzled) receptor genes and two *Lrp* (low-density lipoprotein receptor-related protein) coreceptor genes. Whereas Wnt pathway activity through β-catenin can be

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Box 1. Canonical and noncanonical Wnt signal transduction

Traditionally, the Wnt signaling pathway is divided into a 'canonical' and a 'noncanonical' branch, both of which are activated by the binding of extracellular WNT to Frizzled transmembrane receptors. However, canonical Wnt signaling eventually causes the activation of β-catenin–TCF complexes, whereas noncanonical Wnt signal transduction uses a multitude of different downstream effectors instead [1,53,54].

In the absence of WNT, the rapid turnover of newly synthesized β-catenin is controlled through sequestration of free cytoplasmic β-catenin by the 'scaffolding' complex, consisting of Axin, APC, CK1 (casein kinase 1) and the serine/threonine kinase GSK3. Here, β-catenin is phosphorylated, targeting it for proteasomal degradation. Binding of WNT to Frizzled triggers the recruitment of Dishevelled and Axin by Frizzled and the WNT coreceptor LRP, respectively. Through a so far undisclosed mechanism, which might involve FRAT or GBP proteins in vertebrates, GSK3 is released from the scaffolding complex. As a result, unphosphorylated β-catenin accumulates and interacts with members of the TCF and LEF family of transcription factors to induce transcription of downstream target genes. Interestingly, TCF and LEF proteins act as transcriptional repressors by binding to Groucho proteins in the absence of β-catenin. The ability to switch between repressor and activator status provides tight control over target gene expression.

Initial evidence for the existence of a β-catenin-independent Wnt pathway came from studies in *Drosophila*, where noncanonical Wnt signaling was shown to be required for the establishment of planar cell polarity (PCP), a process in which cells adopt a distinct orientation relative to the plane of the tissue in which they reside [53,54]. A similar pathway also controls polarized cell migration during vertebrate development when so-called convergent extension (CE) movements, in which tissues simultaneously lengthen and narrow, are involved in body axis extension, neural tube closure and tissue morphogenesis [60].

Dishevelled, which is required for both PCP and Wnt signaling through β-catenin, is considered to be the branching point of canonical and noncanonical pathways. At the biochemical level, the events in noncanonical Wnt signal transduction have not yet been fully characterized. In *Drosophila*, the combined actions of flamingo, strabismus, prickle and diego genes induce the asymmetric localization of frizzled and dishevelled complexes at the cell membrane [61,62]. At least some of the vertebrate homologs of these proteins (CELSR, VANGL, Inversin and Diversin) have also been implicated in noncanonical Wnt signaling [63–66]. Downstream effectors of the PCP pathway include small Rho-like GTPases and JNK kinases [67]. However, the precise orchestration of these signaling events is only beginning to be explored. An additional noncanonical branch is the poorly characterized Wnt-Ca^{2+} pathway, in which signaling of WNT–Frizzled complexes through heterotrimeric G proteins and phospholipase C triggers the release of calcium from intracellular storage sites [68]. It remains to be established, however, whether the PCP and Wnt-Ca^{2+} pathways are in fact overlapping or whether context-specific induction of diverse noncanonical pathways can occur (Figure I).

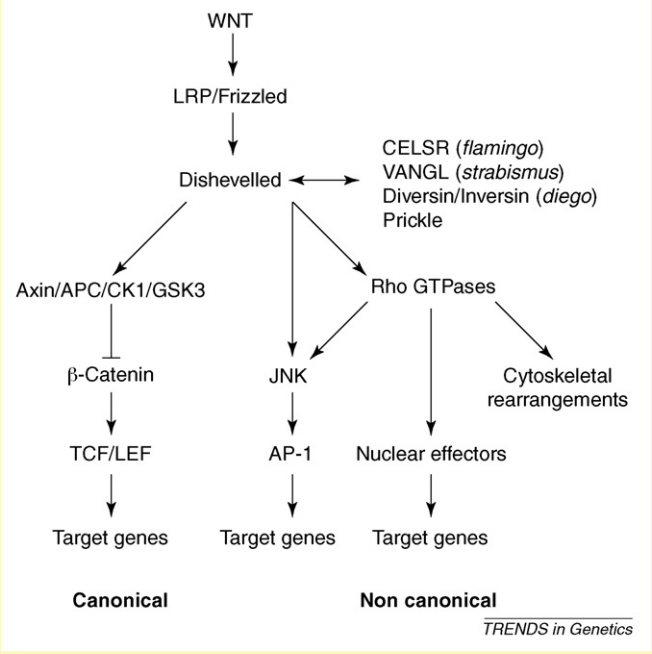

Figure I. Wnt signal transduction pathways. The Wnt signal transduction cascade can be divided into a canonical and a noncanonical branch. The former culminates in the activation of β-catenin–TCF complexes, whereas the latter has been reported to use a multitude of different downstream effectors (see text for details).

Box 2. Model organisms to study Wnt signal transduction

Our ideas regarding Wnt pathway function are largely based on observations made in invertebrate species such as *Drosophila*, or amphibians such as *Xenopus* (previously reviewed in refs [69–72]) (Figure I). Following identification of the murine *Wnt1* gene as an oncogene that contributed to mammary tumorigenesis, it was shown to be homologous to the *Drosophila* segment-polarity gene *wingless*. Genetic epistasis experiments in flies provided the first insight into upstream and downstream signaling events and enabled the discrimination between stimulatory and inhibitory activities. With the capability of rapidly generating large numbers of mutants by both forward and reverse genetics, *Drosophila* has thus been an invaluable tool in delineating the consecutive steps involved in canonical Wnt signal transduction.

Given our profound understanding of *C. elegans* development, this nematode enables the study of Wnt signal transduction in cell-fate decisions, cell migration and polarity in far greater detail than any other organism. However, the *C. elegans* Wnt pathway shows major differences compared with that in flies and vertebrates. At least three β-catenin homologs have been reported, of which only bar-1 functions in canonical Wnt signaling in a complex with pop-1, the *C. elegans* TCF ortholog. To complicate matters further, a fourth β-catenin homolog, sys-1, which bears little if any sequence similarity to the others, was recently reported also to activate downstream target genes upon binding to pop-1, but only in the context of an activated noncanonical Wnt pathway [73]. Rather than helping to elucidate the signaling cascade on the molecular level, as was the case with *Drosophila*, these findings show that the Wnt pathway takes on far more complex forms than we would like to think.

The clawed frog *Xenopus laevis* has revealed the relevance of Wnt signal transduction for the earliest stages of development. A maternal Wnt pathway is crucially required for primary axis specification in *Xenopus*, which is initiated by the stabilization of β-catenin opposite the sperm entry point in the egg, where it leads to the formation of the dorsal organizer [74]. Embryos depleted of canonical Wnt pathway components by the use of antisense oligos fail to establish a normal body axis. Conversely, the injection of mRNAs encoding activators of β-catenin–TCF signaling into the ventral side of early *Xenopus* embryos, where β-catenin is normally degraded, causes body axis duplication. Importantly, Wnt ligands have typically been classified as canonical or noncanonical Wnt components based on their ability to induce such an ectopic axis. More recently, a PCP-like pathway has been shown to function in convergent extension movements and neural tube closure. *Xenopus* thus allows the analysis of both canonical and noncanonical Wnt signaling events during early development, due to the technical ease with which the embryos can be manipulated. On the downside, whereas transient knockdown strategies using morpholino-oligos have been applied successfully, genetic manipulation of the *Xenopus* genome is not possible at the

current time, limiting the range of experimental control. Only recently have transgenic approaches become feasible with a switch from the pseudo-tetraploid *Xenopus laevis* to the diploid *Xenopus tropicalis*, and the first reports of transgenic and inducible constructs, enabling the study of Wnt signal transduction during later stages of development and organogenesis, are now appearing [75,76].

The rise of zebrafish (*Danio rerio*) as a model organism over the last 50 years [77,78] calls into question whether *Xenopus* will hold its own in the era of genetic manipulation. Similar to *Xenopus*, zebrafish embryos develop externally and are more or less transparent, allowing a similar ease of manipulation and monitoring. However, zebrafish have a shorter generation time and are smaller in size, thus enabling the rapid generation of mutant stocks, maintenance of large numbers and the study of adult animals. Moreover, forward genetics using large-scale mutagenesis screens has yielded a wealth of different mutants. The value of zebrafish for the study of Wnt pathway activity is underscored by the identification of *wnt11* as the gene underlying the *silberblick* mutation, which has greatly facilitated the characterization of its role in a CE-like pathway [79], by the fact that mutations in *axin* underly the *masterblind* mutation [80,81], and by the finding that the *headless* mutant harbors defects in *tcf3* [82]. Early zebrafish development is in fact dependent on a maternal Wnt pathway and overexpression of β-catenin induces axis duplication, both of which characteristics bear remarkable similarity to the observations made in *Xenopus* [83]. Together, these properties make zebrafish a promising model organism, destined to become the successor of *Xenopus*. However, tools that enable direct genetic manipulation of the zebrafish genome are not advanced. So far it seems that the generation of knockout animals by targeted mutagenesis remains reserved for mice.

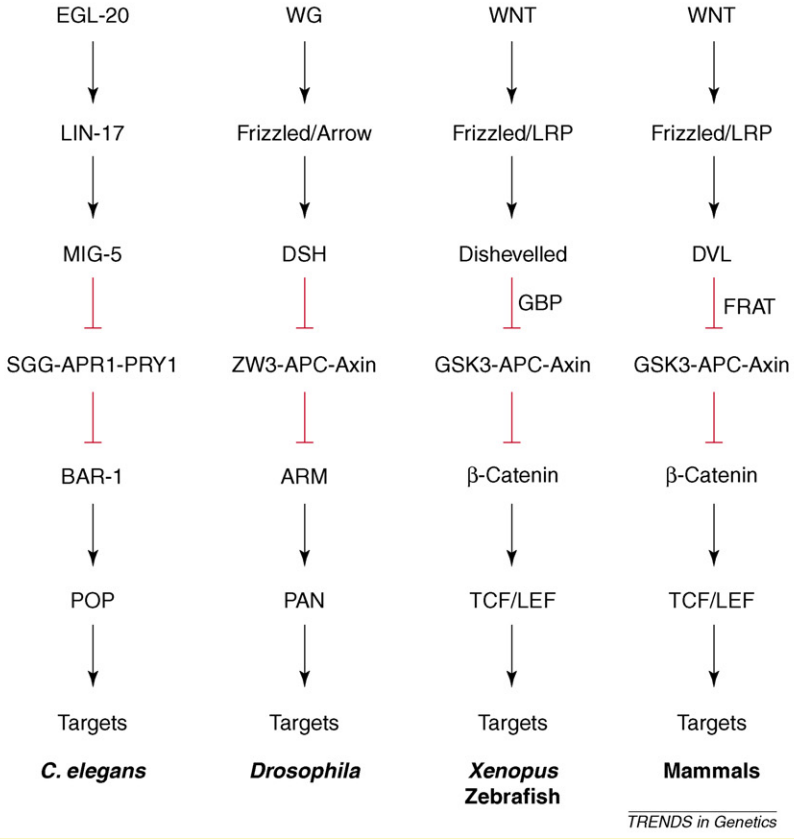

Figure I. Canonical Wnt pathway components show evolutionary conservation across species. A simplified overview of the canonical Wnt signal transduction pathway reveals a striking conservation of core components in a wide range of both invertebrate and vertebrate species.

detected in a broad range of tissues both during development and in adult mice [7], *Wnt* genes themselves often show temporally restricted and highly localized expression patterns. This would seem to indicate that many Wnt family members are involved in similar cellular activities at different sites and times of development. A striking example is the dynamic expression of different *Wnt* genes during embryonic development of the prospective digestive tract [8]. Here, *Wnt4* expression was observed in the intestinal epithelium, whereas *Wnt5A* was only expressed in the mesenchymal compartment. By contrast, *Wnt11* was expressed in the developing epithelium of esophagus and colon in addition to mesenchymal cells in the stomach, whereas expression of *Wnt5B* and *Wnt6* was restricted to the esophageal epithelium. Even at the blastocyst stage of development, locally restricted expression domains have been observed for some of the murine *Wnt* genes [9]. Similar intricate expression patterns have been observed for the *Dkk* (Dickkopf homolog) genes (which encode Wnt inhibitors) during tooth development [10], and for the *Fz* genes during mouse somitogenesis [11]. This agrees with the current opinion that different combinations of WNT–receptor interactions probably govern the complex tissue-specific activities of Wnt signal transduction in addition to the differential activation of canonical and noncanonical downstream signaling events.

In line with their nonoverlapping expression patterns, single-knockout mice often already display dramatic phenotypes (Table 1), many of which cause prenatal or perinatal lethality. These include patterning defects in somites or various organs, some of which reflect those

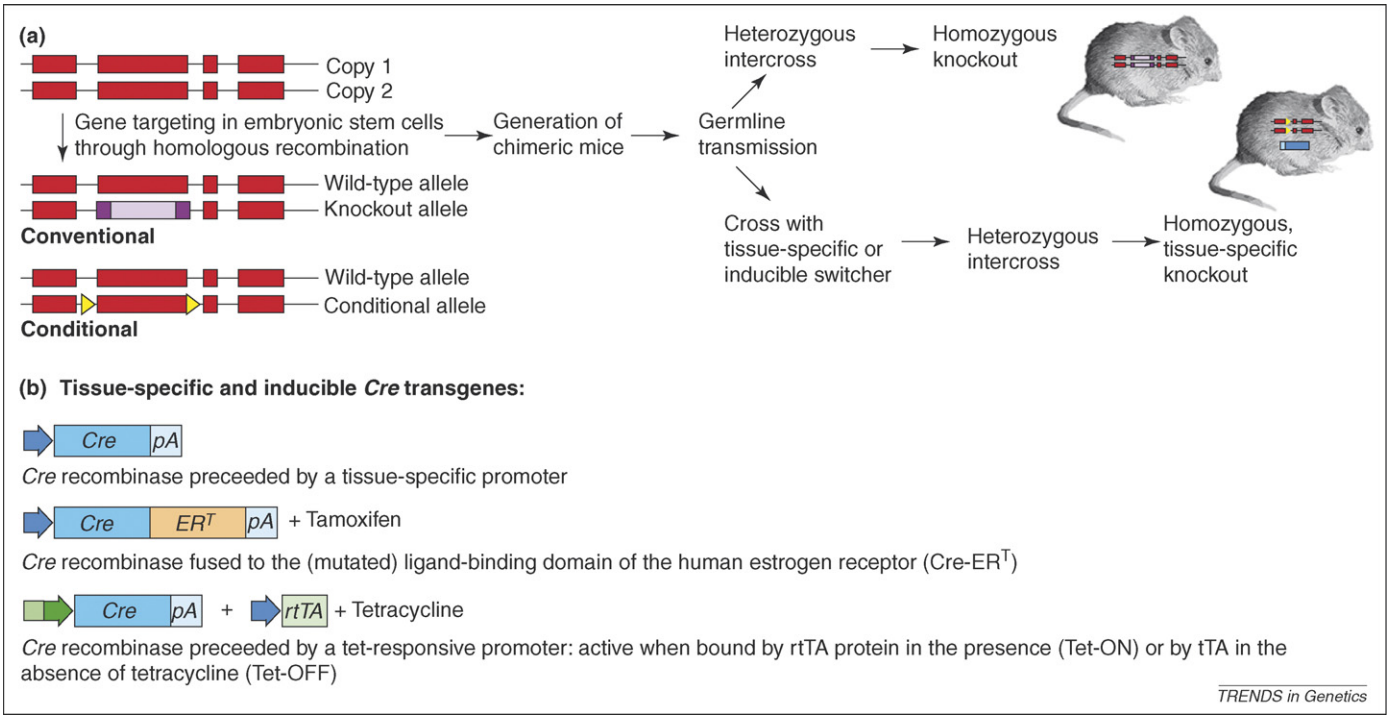

Figure 1. Advances in mouse engineering. **(a)** Knockout mice are generated by gene targeting through homologous recombination in mouse embryonic stem (ES) cells, in which (part of) a gene is either replaced by a selectable marker or flanked by short sequences (most commonly *loxP* sites), which can be recombined by site-specific recombinases such as *Cre*. Germline transmission of the knockout allele eventually enables the generation of homozygous knockout progeny. Importantly, the conditional targeting procedure has to preserve functionality of the gene under study because the conditional allele should behave as wild type. **(b)** Embryonic lethality of conventional knockout mice often precludes the analysis of gene function in later stages of life. Conversely, functional compensation during development can mask knockout phenotypes. Finally, conventional knockouts carry the null allele in all cells of the body, thus preventing the study of wild-type–knockout tissue interactions. By contrast, the use of specific *Cre* mouse strains enables deletion of conditional alleles only when and where the *Cre* recombinase is active. This allows both germline and tissue-specific switching, by using a tissue-specific promoter to drive *Cre* expression. In addition to the somatic application of *Cre* in the form of adenoviral or lentiviral infection, the use of inducible *Cre* transgenic lines gives increased experimental control. The most widely used inducible system uses a *Cre–ER*^{T} fusion protein, in which *Cre* is fused to a gene encoding the (mutated) ligand-binding domain of the human estrogen receptor. *Cre–ER*^{T} is transcribed in all tissues where the promoter is active, but becomes functional only in the presence of tamoxifen, which can be applied topically. Alternatively, expression of the *Cre* transgene can be driven by a tet (tetracycline)-responsive promoter. This approach requires the additional generation of *tTA* or *rtTA* (reverse tTA) activator transgenes. In the Tet-OFF system, *tTA* activates transcription of the *Cre* transgene in the absence of tetracycline (which can be provided in the drinking water). Conversely, in the Tet-ON system the *rtTA* activates transcription of the *Cre* transgene in the presence of tectracycline. For further details see ref. [6].

observed in other model organisms. For example, the hair-patterning defect in *Fz6*-knockout mice reflects planar cell polarity (PCP) phenotypes in the *Drosophila* wing associated with noncanonical Wnt signal transduction. The defects in primary axis formation in *Wnt3* knockouts are reminiscent of the phenotypes observed in *Xenopus* or zebrafish upon ablation of maternal (canonical) Wnt pathway activity.

The existence of some functional redundancy is illustrated by the fact that double-knockout mice often show a more severe phenotype than would be anticipated based on the combined defects present in the single-mutant animals. For example, whereas the phenotype of *Lrp5* knockouts is largely restricted to the bone and *Lrp6*-knockout mice develop to term, *Lrp5–Lrp6* double-knockout mice fail to form a primitive streak and die during gastrulation [12]. Another example of functional redundancy is observed for *Wnt1* and *Wnt3A*, which are coexpressed along the developing dorsal neural tube. *Wnt1–Wnt3A* double knockouts display defects in neural crest development and somite patterning that are not observed in either mutant alone [13,14]. By generating different combinations of compound knockout mice, the possibilities for further study of WNT–receptor interactions are endless.

Knocking out the central players

Whereas mouse knockout studies of the *Wnt* and *Fz* gene families are complicated by the number of homologs and the promiscuity of WNT–FZ interactions, the analysis of downstream Wnt pathway components is obstructed by the fact that multiple upstream signaling events converge on these proteins. Therefore, the observed knockout phenotypes (summarized in Table 2 and Table 3) do not necessarily represent Wnt pathway defects. The latter is illustrated by the phenotype of *Gsk3β* (glycogen synthase kinase-3β) knockout mice. Although GSK3β was known to be involved in a vast array of signal transduction pathways in addition to the canonical Wnt pathway, *Gsk3β*-knockout mice were unexpectedly found to suffer from severe defects in NF-κB (nuclear factor of kappa light chain gene enhancer in B-cells) signaling [15]. Although they opened a new area of research, these data contributed little to our understanding of the *in vivo* effects of GSK3β loss on Wnt signal transduction.

Similar to Wnt proteins and Frizzled proteins, the core components of the canonical Wnt pathway – i.e. Dishevelled, GSK3, APC (adenomatous polyposis coli protein), Axin, β-catenin, TCF (T-cell factor) and LEF (lymphoid enhancer factor) – have multiple homologs, raising the question of possible functional redundancy between family

Table 1. Phenotype of conventional *Wnt-*, *Frizzled-* and *Lrp*-knockout mice

Gene knockout	Phenotype	Refs
Wnt1	Mid- and hindbrain deficiencies	[4,5]
	(hypomorphic allele: *Swaying*)	[85]
Wnt2	Abnormal placental development	[86]
Wnt2B	Viable, no phenotype detected	T. Yamaguchi, personal communication
Wnt3	Failure in A–P axis formation before gastrulation (no primitive streak formed)	[87]
Wnt3A	Truncated A–P axis (loss of caudal somites and tailbud)	[88]
	Disrupted notochord and CNS dysmorphologies	[88]
	Paraxial mesoderm defects	[89]
	Loss of hippocampus	[90]
	Defects in somitogenesis and vertebral patterning	[28]
	Laterality defects	[91,92]
	(hypomorphic allele: *Vestigial Tail*)	
Wnt4	Failure in kidney tubule formation	[93]
	Failure in müllerian duct formation and sex reversal	[94]
	Male gonad determination	[95]
Wnt5A	Truncated A–P axis (with incomplete outgrowth of distal limbs, genitals and tail)	[96]
	Impaired distal lung morphogenesis	[97]
	Abnormal pituitary gland shape	[98]
	Defective pancreatic insulin-cell migration	[99]
Wnt5B	Viable	S. Takada and A. McMahon, personal communication
Wnt6	Viable	A. Kispert, personal communication
Wnt7A	A–P and dorsoventral (D–V) patterning defects with limb and female reproductive tract abnormalities	[100,101]
	Failure in müllerian duct regression in males	[102]
	Delayed synaptic maturation in cerebellum	[103]
	(hypomorphic allele: *Postaxial hemimelia*)	[104]
Wnt7B	Abnormal placental development	[105]
	Lung hypoplasia due to defects in mesenchymal proliferation	[106]
Wnt8A	Viable	T. Yamaguchi and A. McMahon, personal communication
Wnt8B	Viable, no phenotype detected	J. Mason, personal communication
Wnt9A	Skeletal abnormalities and synovial chondroid metaplasia	[107]
Wnt9B	Defects in urogenital development (vestigial kidneys and absence of reproductive duct)	[108]
Wnt10A	Unknown	
Wnt10B	Accelerated myogenic differentiation of myoblasts and increased activation of adipogenic genes upon muscle regeneration	[109]
Wnt11	Kidney hypoplasia due to ureteric branching defects	[110]
Wnt16	Viable	J. Yu and A. McMahon, personal communication
Fzd1	Viable	J. Nathans, personal communication
Fzd2	Viable	J. Nathans, personal communication
Fzd3	Defects in axon tracts in the forebrain	[111]
Fzd4	Progressive cerebellar, auditory and esophageal dysfunction	[112]
	Infertility due to impaired corpora lutea formation and function	[113]
Fzd5	Defects in yolk sac and placental angiogenesis	[114]
Fzd6	Defects in hair patterning	[115]
Fzd7	Viable	J. Nathans, personal communication
Fzd8	Viable	J. Nathans, personal communication
Fzd9	Defects in B-cell development at pre-B-cell stage	[116]
	Hippocampal and visuospatial learning defects	[117]
Fzd10	Unknown	
Lrp5	Defects in cholesterol metabolism and glucose-induced insulin secretion	[118]

Table 1 (*Continued*)

Gene knockout	Phenotype	Refs
	Low bone mass due to decreased osteoblast proliferation, defects in vascular regression	[119]
Lrp6	Truncated A–P axis with loss of caudal somites	[120]
	Neural tube closure defects	[120]
	Mid– and hindbrain deficiencies	[120]
	Reduced production of dentate granule neurons	[121]
	Defects in development of dorsal thalamus	[122]
	Small eyes and aberrant lenses	[123]
	D–V patterning defects with limb abnormalities and discontinuity of the apical epidermal ridge (AER)	[120]
	(hypomorphic allele: *Ringelschwanz*)	[124]

members. Not all homologs have been knocked out. The generation of *Gsk3α*-knockout mice will be of special interest, given how surprisingly little is still known about the specificities of the α and β isoforms of GSK3. Hopefully, *GSK3α*-deficient mice will answer some pressing questions regarding the role of GSK3α in Wnt signal transduction. Likewise, although the APC tumor suppressor protein is arguably the most studied component of the Wnt pathway, the endogenous function of its close homolog APC2 is still unresolved. It will be interesting to see whether loss of APC2 results in a phenotype of similar severity as that of *Apc*-knockout mice, which fail to undergo proper gastrulation [16]. Whereas overexpression of mammalian *Apc2* can substitute for the loss of APC with regard to the down-regulation of β-catenin [17], the expression of murine *Apc2* is restricted to the nervous system [18], and reports on the loss of *APC2* in tumors remain scarce [19,20]. Although initial studies in *Drosophila* also suggested that *apc* and *apc2* had different functions, as illustrated by the differences in tissue distribution, subcellular localization and single-knockout phenotypes, the combined inactivation of the two homologs revealed extensive functional redundancy [21–23].

Interestingly, mice deficient for FRAT (frequently rearranged in advanced T-cell lymphomas), a GSK3-binding protein postulated to bridge signaling from Dishevelled to GSK3, are normal and display no obvious defects in signaling through β-catenin and TCF [24]. In stark contrast, depletion of the Frat homolog Gbp (Gsk3-binding protein) in *Xenopus* prevents endogenous axis formation in the developing embryo [25]. This discrepancy underscores the danger of extrapolating observations made in a single model organism.

Table 2. Phenotype of conventional knockout mice deficient for intracellular canonical Wnt pathway components

Gene knockout	Phenotype	Refs
Dvl1	Abnormal social interaction	[26,125]
Dvl2	Cardiovascular outflow tract defects (due to defect in cardiac neural crest development)	[27]
	Vertebral and rib malformations (due to defects in somite segmentation)	[27]
	Neural tube closure defects	[27]
Dvl3	Unknown	
Frat1–2-3	No obvious abnormalities or defects in Wnt signal transduction	[24]
Axin1	A-P axis duplication	[51]
	Neuroectodermal and cardiac abnormalities	[51]
	(as observed in the mutant allele *Fused*; no conventional knockout has been generated)	
Axin2	Malformation of skull due to increased osteoblast proliferation and differentiation	[52]
Gsk3α	Unknown	
Gsk3β	Liver degeneration due to defects in NFκB signaling with increased TNF-α (tumor necrosis factor α) toxicity	[15]
Apc1	Development of primitive ectoderm fails before gastrulation	[16]
	(Partial) A–P axis duplication, anterior truncation and defects in ventral morphogenesis (in mice homozygous for a hypomorphic allele)	[126]
	(mutant allele *Min* leads to the development of intestinal polyps in heterozygotes with loss of heterozygosity in polyps)	
Apc2	Unknown	
β-catenin	Failure in A–P axis formation before gastrulation (no primitive streak formation, defects in embryonic ectoderm)	[38,127]
Lef1	Defects in pro-B-cell proliferation and survival	[48]
	Defects in the formation of many organs that require inductive tissue interactions (such as teeth, mammary glands, whiskers and hair)	[128]
	Brain abnormalities ranging from loss of dentate gyrus granule neurons to complete absence of hippocampus	[129]
Tcf1	Progressive defects in thymocyte proliferation and differentiation	[45,130]
Tcf3	Failure in A–P axis formation (expanded or duplicated axial mesoderm and A–P structures, including node and notochord)	[41]
Tcf4	Depletion of epithelial stem-cell compartments in the intestine	[131]

Table 3. Phenotype of conventional knockout mice deficient for noncanonical Wnt pathway components

Gene knockout	Phenotype	Refs
Celsr1	Misorientation of inner ear hair cells and neural tube defects (observed in the *crash* and *spin cycle* mutants)	[63]
Celsr2	Unknown	
Celsr3	Defects in axon tract development	[132]
Vangl1	Unknown	
Vangl2	Neural tube closure defects Misorientation of inner ear hair cells Cardiovascular outflow tract defects (observed in the *looptail* mutant)	[133–136] [135] [31]
Prickle[a]	Unknown	[137]
Ankrd6 (Diversin)	Unknown	
Inversin	Reversal of left–right asymmetry and kidney cysts (observed in the *inv* mutant)	[138]

[a]Three murine Prickle homologs have been identified, named Prickle, Dyxin and Testin, none of which have been characterized extensively.

Dishevelled: at the crossroads of canonical and noncanonical pathways

In the case of Dishevelled (DVL), functional redundancy has been revealed by the severe phenotype of *Dvl* compound-knockout animals compared with that of the single knockouts. For instance, 2–3% of mice deficient for *Dvl2* have thoracic spina bifida, whereas this is never observed in *Dvl1*-knockout mice [26]. By contrast, virtually all *Dvl1–Dvl2* double-knockout mice have a completely open neural tube from midbrain to tail, suggesting that DVL is required for neural tube closure [27]. It will be interesting to see whether the phenotype of *Dvl1–Dvl2* double-knockouts is further aggravated in the context of *Dvl3* deficiency.

Given that DVL functions at the crossroads of canonical and noncanonical Wnt signaling, it is to be expected that the phenotypes observed in *Dvl*-knockout mice sample both of these pathways. Indeed, vertebral and rib malformations are reminiscent of the somite segmentation defects found in *Wnt3A* hypomorphs [28] and are thought to reflect defects in canonical Wnt signal transduction. By contrast, severe neural tube closure defects, such as the craniorachisis observed in *Dvl1–Dvl2*-knockout mice, are more reminiscent of the phenotype observed in mice deficient for typical noncanonical Wnt pathway components. Both the *looptail* and *crash* mouse mutants, which harbor mutations in the *Vangl2* (van gogh-like 2) and *Celsr1* (cadherin EGF LAG seven-pass G-type receptor 1) genes respectively, have convergent extension defects resulting in an inability of the neural folds to close, similar to the situation observed in the *Dvl1–Dvl2* knockout [29]. In addition, the cardiac outflow deficiencies that have been described in *Dvl2*-knockout mice can be attributed to defects in canonical and noncanonical pathways. A similar phenotype has been described in the *looptail* mouse [30,31], but has also been suggested to involve signaling through GSK3 and β-catenin, leading to induction of the PITX2 (paired-like homeodomain transcription factor 2) transcription factor [32].

Backups for β-catenin

Although canonical Wnt signal transduction is generally considered to culminate in the activation of β-catenin–TCF complexes, little is still known about possible functional redundancy between β-catenin and its family members. In addition to β-catenin, at least two other armadillo repeat-containing proteins have been implicated in Wnt signal transduction in mammals. These are γ-catenin, also known as plakoglobin, and p120-catenin, both of which also function in cell adhesion complexes, as does β-catenin. Plakoglobin can bind to TCF and LEF factors in human embryonic kidney cells and can function as a transcriptional activator in this context [33]. However, it has also been shown to bind to a different site on TCF4 than β-catenin and has been postulated to inhibit the activity of β-catenin–TCF4 complexes in this way [34].

Plakoglobin-knockout mice die due to defects in cardiac development [35,36] and, depending on the genetic background, can have severe skin blistering [36]. Both of these phenotypes were shown to be caused by defects in cell adhesion and were found to arise only later during embryonic development, suggesting that β-catenin, or an unrelated protein, was able to compensate for the loss of plakoglobin up to a certain point. Indeed, increased β-catenin was found to be present in desmosomal junctions in plakoglobin-null mice, whereas it is normally restricted to other adherens junctions [37]. Similarly, plakoglobin can be found in the adherens junctions of β-catenin-deficient mice [38]. Thus, although these observations indicate that β-catenin and plakoglobin are, at least partially, functionally redundant in cell adhesion, the relative contribution of plakoglobin to canonical Wnt signal transduction remains unknown.

Unexpectedly, p120-catenin–Kaiso complexes were recently shown to act in parallel to β-catenin–Tcf complexes in *Xenopus* [39]. Like Tcf, Kaiso can function as a transcriptional repressor. Moreover, Tcf and Kaiso cooperate in the repression of target genes. Similar to the way in which binding of β-catenin transforms Tcf into a transcriptional activator, p120-catenin was shown to relieve Kaiso repressor activity. Kaiso-deficient mice were recently reported to display no overt phenotypic abnormalities, although they did exhibit delayed intestinal tumorigenesis when crossed with $Apc^{+/Min}$ mice [40]. It will be interesting to see whether p120-catenin-knockout mice, whose generation has not yet been reported, have defects in Wnt signal transduction, cell adhesion or a combination of both. In addition, the observed interaction between p120-catenin and Kaiso also serves as a reminder that so far unidentified transcription factors might associate with plakoglobin as well.

TCF and LEF: activators or repressors?

Mouse knockout studies have revealed the importance of TCF and LEF repressor functions. Whereas mice deficient for *Tcf1*, *Lef1* or *Tcf4* survive to term, *Tcf3*-knockout mice show partial axis duplication, which becomes apparent at the time of gastrulation [41]. Because this phenotype is similar to that of *Axin*- or *Apc*-knockout mice, this observation indicates that TCF-mediated repression of

target genes has an important role in embryonic axis formation. By contrast, formation of the primitive streak, which is dependent on Wnt signaling activity through β-catenin and which is compromised in mice deficient for *Wnt3*, *Lrp5–Lrp6* or β-catenin, is completely normal in *Tcf3*-knockout mice, as is the activity of an *in vivo* β-catenin–TCF reporter construct [41]. Because *Tcf4* is normally not expressed in pregastrulation embryos, this would suggest that LEF1 and TCF1 are the sole candidates for generating activator complexes with β-catenin during anterior–posterior (A–P) axis formation. However, both *Lef1* and *Tcf1* single-knockout mice, in addition to *Lef1–Tcf1* double-knockout mice, can still form a normal A–P axis. In fact, *Lef1–Tcf1* compound-knockout mice have a phenotype that is similar to *Wnt3A* knockouts, with paraxial mesoderm defects and loss of caudal somites [42]. In addition it phenocopies defects observed in *Wnt2*- and *Wnt7A*-knockout mice, with defects in placenta formation and limb development, respectively. These observations suggest the presence of another, as yet unidentified, transcription factor that is responsible for the activation of the observed β-catenin–TCF reporter activity.

Future directions and perspectives: 21st century mice

In summary, mice deficient for canonical and noncanonical Wnt pathway components have yielded unprecedented insights into the role of Wnt signal transduction in higher vertebrates. Whereas some knockouts have confirmed our expectations, others have produced unexpected results, generating new questions that are now awaiting an answer (Box 3).

Currently, much of the data distilled from these conventional knockout mouse models are descriptive. Given the large number of proteins involved in both the canonical and the noncanonical Wnt pathways, a gigantic undertaking is needed before we can provide the full atlas of Wnt-dependent processes in mammalian development and physiology. To further our understanding of Wnt signaling *in vivo*, we will now have to take advantage of the availability of the second generation of mouse models, which give increased experimental control.

In many cases, early (embryonic) lethality and severe developmental defects (summarized in Figure 2) have precluded additional analyses. The possibility to generate conditional and inducible knockout and transgenic mice now enables investigators to switch genes on and off exclusively in the tissue and at the time of interest, thus enabling the study of Wnt signal transduction in later stages of development or in more defined settings. Unfortunately, an overview of the Wnt-directed conditional mouse models generated to date is beyond the scope of this review. However, their usefulness is exemplified by the phenotype of conventional *Wnt3*-knockout mice, which fail to form a primitive streak and are unable to complete gastrulation. Conditional ablation of *Wnt3*, however, revealed a role for WNT3 in limb patterning at the apical epidermal ridge [43], in agreement with the observation that defects in WNT3 underlie a familial case of tetra-amelia, a rare human genetic disorder in which all four limbs are absent. Remarkably, the conditional ablation of the β-catenin gene in the hematopoietic compartment revealed a far less crucial requirement for β-catenin in the processes of hematopoietic stem cell renewal and lineage differentiation than expected [44], thus calling for a β-catenin-independent explanation of the phenotypes of *Lef1–Tcf1*-knockout mice [45–48] and questioning the importance of canonical Wnt signal transduction for hematopoietic stem cell maintenance [49] under steady-state conditions. Importantly, knockout studies have so far also largely ignored the fact that Wnt signal transduction is important for tissue interactions. Disrupting the pathway in a systemic fashion, probably has different results from ablating gene function only in, for example, epithelial or mesenchymal cells.

Another pressing issue is the question of what is the extent of functional redundancy between family members. The systematic generation of knock-in mice might provide an answer. *Axin2*, for example, which is normally expressed at restricted sites, can complement for the loss of *Axin1* when it is knocked-in to the *Axin1* locus [50]. However, given the divergent phenotypes of *Axin1*- [51] and *Axin2*-deficient mice [52], they normally are unable to compensate fully for each other.

Knock-in studies will also be of special interest for the TCF and LEF transcription factors. Distinguishing between the importance of their repressive and activating roles during mammalian development will only be possible by generating mice that carry mutant alleles in the endogenous locus, lacking either the activator or repressor functions. The construction of a *Tcf3ΔN* knock-in mouse, for instance, in which possible activator functions are abolished by deletion of the β-catenin binding site, will answer whether TCF3 functions as a repressor during A–P axis formation and whether it is in fact ever required as a transcriptional activator.

Similarly, the generation of an allelic series might also be helpful in explaining other unexpected observations. For instance, the fact that *Vangl2* and *Celsr1* mutants display phenotypes similar to mice that lack *Dvl1–Dvl2* (proposed activators of noncanonical Wnt signaling), is not easily reconciled with the inhibitory role of their *Drosophila* orthologs in PCP-signaling [53,54] (Figure 2). This would seem to imply that noncanonical Wnt signaling activities are dependent on a delicate balance, with distortions in either direction resulting in the reported malformations. A possible way to address this hypothesis would be to generate mutant alleles encoding proteins that are defective in interacting with some, but not other, binding partners or that can no

Box 3. Questions outstanding

- Wnt signaling in the intestinal epithelium is required not only for the maintenance of undifferentiated progenitors, but also for driving a specific Paneth-cell differentiation program [84]. Does Wnt signaling have such a dual role in other tissues as well?
- In what context does Wnt signaling result in cell proliferation rather than differentiation? Accumulating evidence suggests that the effects of Wnt signaling on proliferation versus differentiation might be temporally regulated within the same tissue. Does the timing of Wnt pathway (in)activation result in different phenotypic outcomes?

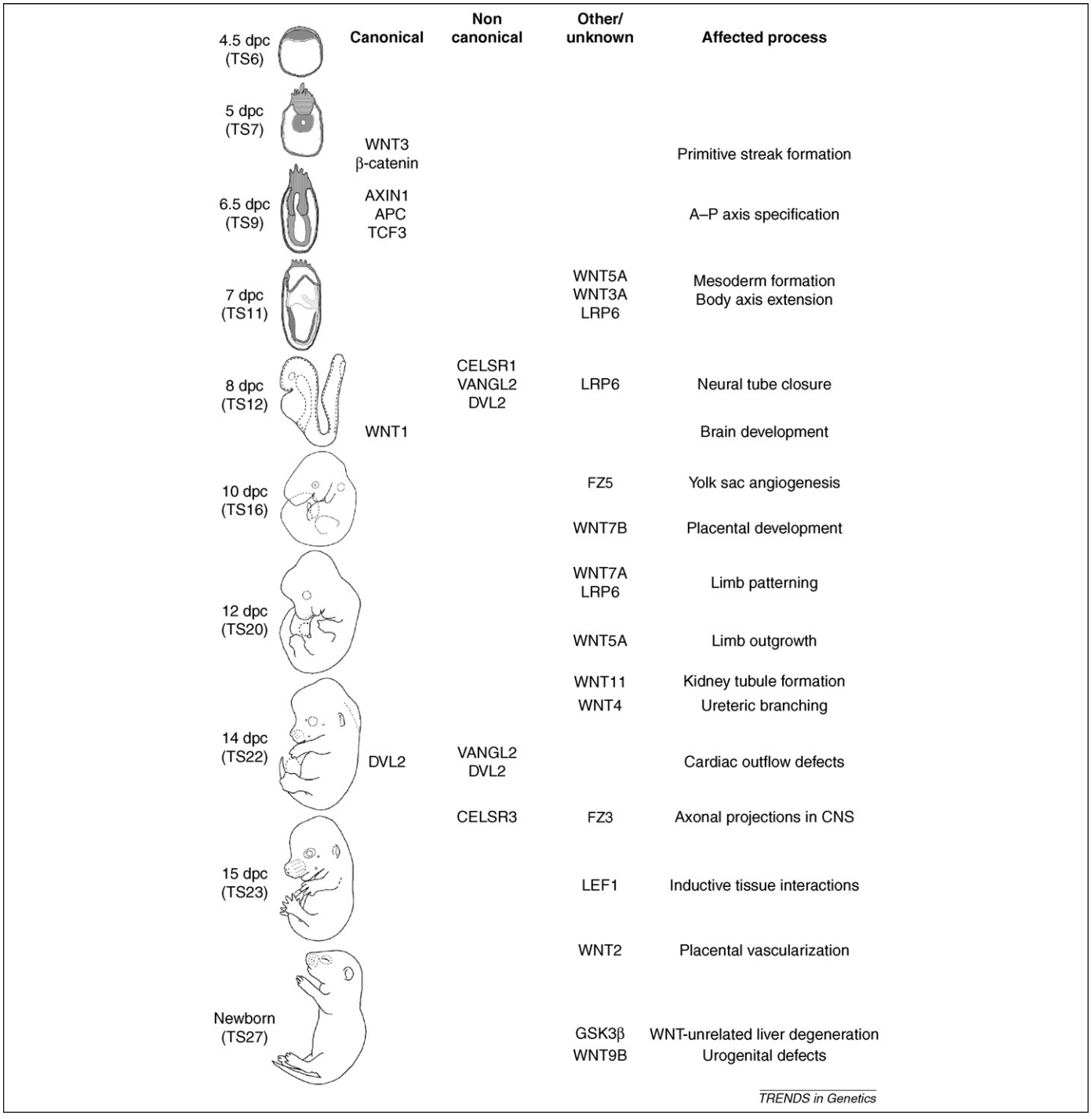

Figure 2. Overview of the developmental phenotypes observed in conventional knockout mice with targeted mutations in canonical and noncanonical Wnt pathway components. Mice deficient for core components of the Wnt signal transduction pathway often display dramatic phenotypes resulting in prenatal or perinatal lethality. Embryonic development proceeds normally through the blastocyst (4.5 dpc) and egg cylinder (5 dpc) stages. The earliest developmental phenotypes are observed before gastrulation (6.5–7 dpc), because the primitive streak fails to form in *Wnt3*$^{-/-}$ and *β-catenin*$^{-/-}$ mice. The depicted phenotypes sample both canonical and noncanonical Wnt pathway defects. In the absence of decisive evidence that the observed phenotypes result from disturbed β-catenin–TCF activity, they cannot be attributed to the canonical pathway with any certainty. These include many of the observed Wnt and Frizzled phenotypes, because the activities of these proteins are shared by canonical and noncanonical Wnt pathways. TS, Theiler stage; dpc, days postcoitum. The cartoons depicting developmental stages were taken with permission from the Edinburgh Mouse Atlas Project (http://genex.hgu.mrc.ac.uk) and are not to scale.

longer be properly localized within the cell. Obviously, the mutations generated in such an allelic series should be as defined as possible to minimize the chances of disrupting the expression levels of the gene of interest. Recently, an allelic series of *Dvl2* BAC (bacterial artificial chromosome) transgenes was analyzed for their capacity to rescue the neurulation defects observed in *Dvl1–Dvl2* double-knockout mice, demonstrating the power of such an approach [55].

Concluding remarks

It is not yet possible to attribute many of the observed developmental phenotypes to either canonical or noncanonical pathway defects (Figure 2). This holds true not only for

the WNT ligands, but also for the FZ and LRP (co-)receptors. A final verdict will have to await experiments such as those, for example, that have been performed using mice carrying a conditional β-catenin allele and a *Wnt1–Cre* transgene [56]. The brain malformations observed in these mice reflect the phenotype of conventional *Wnt1*-knockout mice [5], thus enabling this to be classified as a canonical pathway defect. In addition, knockout mice could be backcrossed to canonical Wnt-pathway reporter mice, such as the *BATGAL* [7] and *TOPGAL* [57] mice (which have a β-galactosidase transgene under the control of a LEF/TCF and β-catenin-inducible promoter), or *Axin2-lacZ* [58] mice (which have a β-galactosidase gene knocked in to the *Axin2* locus), to determine whether gene ablation has affected the endogenous pattern of β-catenin–TCF activity.

Finally, it should be noted that even the analysis of the conventional knockout models generated to date is still incomplete. In the absence of standardized phenotyping efforts, the study of any mutant is seldom exhaustive. In addition to studying metabolic and behavioral parameters, new noninvasive imaging techniques such as MRI (magnetic resonance imaging) or PET (positron emission tomography) scan analysis, can be expected to increase markedly the informative value of these mice. Furthermore, refined experimental approaches can circumvent some of the observed perinatal lethality. A recent good example was a study in which tissues from *Wnt5A*- and *Wnt7A*-mutant mice were studied in kidney capsule transplantation experiments, revealing a role for both genes in uterine gland development [59]. Finally, the incorporation of genomic-scale approaches, including transcriptional profiling and proteomic analysis, on knockout tissues could begin to reveal the mechanisms behind the observed phenotypes. In light of the emerging complexity of the Wnt signaling pathway, which includes functional redundancy due to extended gene families and backup networks, we still face a daunting task to reach a full understanding of Wnt signal transduction *in vivo*.

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