EMBO Workshop on Wnt Signalling

Stem Cells | Development | Disease

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"**Wnters Down Under**"

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Meeting Report Summary
Every year or so an international assembly of scientists meet with the aim of better understanding the mechanisms of Wnt signalling and the role this pathway plays in normal and disease contexts. Wnt signalling encompasses so many fields that more recent Wnt meetings tend to have some sort of focus. The broad focus of the inaugural Australian Wnt meeting was signalling mechanisms, adult stem cells and cancer. The culture of the international Wnt meetings is to present the latest, often unpublished, results which generates vibrant discussions and advances the field at a phenomenal rate. Now that these discoveries have been published, some highlights from the Australian Wnt meeting are summarised here. The highlights include, identification of a multipotent adult mammary stem cell; characterisation of the context dependent nature of Wnt signalling; the intricacies of achieving the "just right" level of Wnt signalling that drives efficient adenoma formation – the "Goldilocks" effect; several "bench to bedside" reports on therapeutic targeting via Wnt pathway components, especially the Frizzled receptors but also the intracellular components. This meeting report provides a snap-shot in time for the Wnt signalling field and documents the extraordinary advances made in this vibrant field from one meeting to the next.

Introduction
The international Wnt meetings bring together students, scientists and clinicians from diverse fields to discuss the latest discoveries. The 2014 EMBO Workshop on "Wnt Signalling: Stem Cells, Development, Disease" was held on Broome's magnificent Cable Beach in Western Australia. This was the first EMBO workshop held in Australia, and the first time that the Wnt meeting has been held anywhere in the Southern Hemisphere. It was a truly global Wnt meeting with approximately 2/3 of the 130 delegates coming from the US/Europe/UK and 1/3 from Australia/Asia. Keeping in the spirit of these meetings, much of the work that was presented was unpublished and thus could not be "pre-published" in a meeting summary. Now that most of what was presented at the meeting has been published, some of the highlights have been summarised in this meeting report.

Meeting Summary
The meeting was opened with the EMBO keynote delivered by Hans Clevers (Hubrecht Institute, Utrecht, the Netherlands). He reported on the discovery of leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5) as a stem cell marker and functional receptor of the Wnt agonist R-spondin in adult stem cells, and the advances this discovery has made to regenerative medicine and anti-cancer treatment. He reported on using 3-dimensional (3D) organoid cultures derived from healthy and tumour tissue of colon cancer patients for a high throughput drug screen to identify gene/drug associations that may facilitate personalized therapy [1]. The keynote was followed by the first of two vibrant poster sessions. Over the next 3 days, we heard the latest findings in Wnt signalling mechanisms, stem cell biology, development and disease. Irrespective of the session title, be it cancer/disease, stem cells or development, there was a common theme of discovering novel therapeutic avenues for drug development to treat human diseases. Also, more evidence that Wnt signalling is exquisitely regulated at each step of the pathway and that the level of Wnt signalling is critical in Wnt-driven processes. Here are some highlights of this Wnt meeting.

***Receptor-ligand*:** Unexpected functional insight into Wnt signal inhibition by the secreted Wnt-inhibitory enzyme Notum was presented. This enzyme had previously been thought to act as a phospholipase that cleaves the glycosylphosphatidylinositol (GPI) anchors of glypicans, thereby shedding Wnt molecules from the cell surface. Matthias Zebisch (University of Oxford, UK) presented a different view resulting from a collaboration between the structural biology laboratory of Yvonne Jones (University of Oxford, UK) and the developmental biology group of Jean-Paul Vincent (MRC National Institute for Medical Research, London, UK). He showed that Notum does interact with glypicans and depends on them to antagonise Wnt signalling. However, Notum does not seem to cleave glypicans. Instead, it appears to be a secreted protein deacylase with specificity for the C9-C10 unsaturated lipid (palmitoleate) tails of Wnts. This explains Notum's ability to suppress Wnt signalling since the lipid moiety is essential for binding to Frizzled. Structural data was presented that showed palmitoleate binding in a hydrophobic active site cavity in the Notum core and binding of heparan sulfate mimics to the protein surface [2]. Xi He (Harvard Medical School, Boston, USA) discussed another family of Wnt inhibitors with catalytic capacities, the TIKI family of metalloproteinases that inhibit Wnt signalling by cleaving the Wnt-amino terminal region of specific Wnt ligands, and shed light on the substrate specificities of TIKI1/2 [3].

Tao-Hsin Chang (Yvonne Jones lab, University of Oxford, UK) presented the structures of human Norrin and its complex with human Frizzled4 cysteine-rich domain (Fz4-CRD), plus unliganded structure of Fz4-CRD. These structures together with biophysical and cellular assays provide a framework to explain numerous disease-related mutations in triggering signal transduction. The complex architecture and conserved amino acids in the interaction interface provide important insights for understanding the binding specificity of Frizzleds and developing therapeutic strategies [4].

Dianqing (Dan) Wu (Yale School of Medicine, Newhaven, USA) described the recent work on how Wnt stimulates the formation of phosphatidylinositol (4,5)bisphosphate [PtdIns (4,5)P2] and how PtdIns (4,5)P2 regulates the formation of low-density-lipoproteinreceptor-related protein 6 (LRP6) signalosome formation. Wnt3a-induced PtdIns(4,5)P2 promotes the assembly of LRP6 signalosomes via the recruitment of adaptor protein 2 (AP2) and clathrin, and LRP6 signalosomes are localized at cell surfaces rather than being internalized. However, rapid PtdIns(4,5)P2 hydrolysis induced artificially after WNT3A stimulation could lead to marked LRP6 internalization. Nevertheless, this result indicates that LRP6 internalization may not be a prerequisite for Wnt signalling to β-catenin stabilization [5]. In addition, new insights into how Dvl interacts with and activate phosphatidylinositol-4-phosphate 5-kinase type I (PIP5K1) were discussed based on new structural and mutagenesis studies [6][7].

John McAvoy (University of Sydney, Australia) presented work that demonstrates the Wnt-Frizzled/Planar Cell Polarity signalling regulates the polarised behaviour of fibre cells that determines lens three-dimensional cellular architecture. How a particular tissue or organ develops its characteristic three-dimensional cellular architecture is often a poorly understood part of its developmental program; yet precise regulation of these features can be critical for function. The lens of the eye illustrates this well because it needs to develop very precise dimensions and curvature to do its job of focussing light onto the retina. He described a fibroblast growth factor (FGF)-activated mechanism intrinsic to the lens that involves interactions between the Wnt-Fz/Planar Cell Polarity and Jagged/Notch signalling pathways. This reciprocal epithelial-fibre cell interaction is critical for the assembly and maintenance of the highly ordered three-dimensional architecture that is central to lens function [8].

***Cytoplasm/nucleus*:** Activity of the Wnt pathway is based on the cytosolic level of β-catenin, which is regulated by the destruction complex consisting of Axin, Adenomatous Polyposis Coli (APC), glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1). Axin is the rate-limiting factor of the destruction

complex stability and its levels can be regulated through poly-ADP-ribosylation by tankyrases (TNKS1/2). Inhibition of the activity of TNKS1/2 leads to increased levels of Axin and reduced Wnt/β-catenin signalling. Tor Espen Thorvaldsen from Harold Stenmark's lab (Institute for Cancer Research, Oslo, Norway) showed that incubation of colon cancer cells with a small molecule tankyrase inhibitor (G007-LK) induces the appearance of large perinuclear protein clusters that contain all the components of the destruction complex, in addition to ubiquitin. Structured illumination and electron microscopy revealed that TNKS inhibition attenuates WNT/β-catenin signalling by promoting dynamic assemblies of functional, active destruction complexes into a TNKS-containing scaffold even in the presence of an *APC* truncation that constitutively activates Wnt signalling [9].

Christof Niehrs (DKFZ-ZMBH Alliance, Heidelberg, Germany) introduced a new branch of canonical Wnt signalling, Wnt-dependent stabilization of proteins (Wnt/STOP), which is independent of β-catenin and peaks during mitosis. Wnt/STOP plays a critical role in protecting proteins, including c-MYC, from GSK3-dependent polyubiquitylation and degradation. STOP signalling increases cellular protein levels and cell size, to promote cell growth. Wnt/STOP, rather than β-catenin signalling, is the dominant mode of Wnt signalling in several cancer cell lines, raising potentially new perspectives for cancer treatment [10].

Mariann Bienz (MRC Laboratory of Molecular Biology, Cambridge, UK) focused on the function of the Pygopus (Pygo)-BCL9 complex in Wnt-dependent transcription. Marian reported that Drosophila Pygo is required for early embryonic development, before the onset of Wingless signalling, consistent with the proposed function of Pygo as an anti-repressor. She outlined a model according to which this factor uses its N-terminal asparagine proline phenylalanine (NPF) motif to associate with Wnt-responsive enhancers, and that it primes the linked genes for subsequent Wnt-dependent transcription by capturing Armadillo/β-catenin through the BCL9 adaptor and thus enabling it to overcome Groucho-dependent repression[11].

Using a mouse model of intestinal cancer in which adenoma formation is initiated by Cre-recombinase-mediated truncation of *APC*, Alan Clarke (Cardiff University, Wales, UK) demonstrated that the chromatin remodelling factor Brahma-related gene 1 (BRG1) is necessary for Wnt-driven gene expression changes that initiate and maintain adenoma formation. He proposed a "just right" level of Wnt-driven transcription for adenoma formation or the "Goldilocks" effect. Brg1 therefore constitutes a potential therapeutic target in cancers with an aberrantly activated Wnt pathway [12]. Work from Trevor Dale (Cardiff University, Wales UK), expanded on previous work from his collaboration with Alan Clarke's laboratory (Cardiff University) on the role of Axin1 in liver cancer [13] to the role of Axin2 and APC. The oncogenic role of Axin1, Axin2 and APC were compared and contrasted to shed light onto the molecular mechanisms underlying Wnt activation and chromosomal instability.

Beric Henderson (University of NSW, Australia) revealed new insights into the intracellular trafficking, dynamics and function of β-catenin and APC. Live cell imaging showed dynamic movement of APC and β-catenin, correlating their trafficking with novel protein interactions, functions and cellular processes. Nuclear entry of β-catenin was mediated by its central armadillo repeats and flexible "tail" sequences, through direct interaction with the nuclear pores via hydrophobic peptide regions [14]. In addition, a series of novel protein interactions that link APC to specific functions at the centrosome and mitochondria were presented [15, 16]. The continuing discovery of unique and overlapping cellular locations for APC and β-catenin provides a unique view into the dynamic nature of protein signalling, and the critical importance of intracellular trafficking for protein regulation.

Marian Waterman (University of California, Irvine) probed the connection between Wnt and metabolism using biochemical methods and state-of-the-art, non-invasive fluorescence imaging to monitor NADH in living cells (known as FLIM for Fluorescent Lifetime Imaging Microscopy). FLIM

measurements showed that Wnt promotes a shift to glycolysis *in vitro* as well as *in vivo* in tumours. This work identified pyruvate dehydrogenase kinase 1 (PDK1) and others as Wnt target genes. PDK1 expression rescued dnLEF/dnTCF [dominant-negative (dn) lymphocyte enhancer factor (LEF)/dn T-cell factor (TCF)] interference and restored aerobic glycolysis to tumour cells (Warburg metabolism). FLIM was also used to discover that these activities correlate with normal modes of stem cell metabolism in the intestine, a gradient or "Metabolic Trajectory" wherein $Lgr5^{+}$ stem cells and Paneth cells at the base of crypts are glycolytic and differentiated cells at the mucosal surface utilize oxidative phosphorylation. In addition, glycolytic cells signal to their environment to promote vessel development, a signal that promotes angiogenesis in tumours. In normal intestine this correlates with a strong, dense network of vessels that completely surround glycolytic stem cells. Thus, the Warburg shift to glycolysis that helps colon cancer cells withstand hypoxia and the stress of nutrient limitation appears to derive from normal functions of Wnt signalling in intestinal stem cells [17].

Patrick Tam (Children's Medical Research Institute, University of Sydney, Australia) highlighted the functional attribute of the transcription factor, orthodenticle homeobox 2 (Otx2), in the modulation of canonical WNT signalling activity. Otx2 acts upstream to regulate the expression of Dickkopf1 (Dkk1), a WNT antagonist, and another transcription factor, LIN-11-Isl1-MEC-3 (LIM) homeobox 1 (Lhx1), which in turns controls the expression of Dkk1 and three other negative regulators of WNT activity. The intersection of Otx2 and Lhx1 with WNT signalling therefore underpins the molecular control of head morphogenesis during early mouse development [18, 19].

Stefan Hoppler (University of Aberdeen, UK) highlighted the context-dependent nature of Wnt signalling. A direct comparison of genome-wide occupancy of β-catenin with a stage-matched Wnt-regulated transcriptome revealed that Wnt signalling regulates β-catenin binding to Wnt target genes not only when they are transcriptionally regulated, but also in contexts in which their transcription remains unaffected. Thus, the transcriptional response to Wnt signalling depended not only on recruiting β-catenin but also on additional factors, such as bone morphogenetic protein (BMP) or FGF signalling which do not influence recruitment. This context specificity of Wnt-regulated transcriptional mechanisms provides a mechanism for the tissue-specific functions of Wnt/β-catenin signalling in embryonic development but also for stem cell-mediated homeostasis and cancer. This work proposes that chromatin association of β-catenin alone does not necessarily identify transcriptionally Wnt-regulated genes. Context-dependent mechanisms are crucial for transcriptional activation of Wnt/β-catenin target genes subsequent to β-catenin recruitment [20].

Terry Yamaguchi (NIH, Frederick, USA) showed in mouse embryos and differentiating embryonic stem cells that the specificity protein (Sp) 1/Klf-like zinc-finger transcription factors Sp5 and Sp8 (Sp5/8) are gene-specific transcriptional coactivators in the Wnt/β-catenin pathway. Sp5/8 bind directly to Wnt target gene enhancers and to adjacent, or distally positioned, chromatin-bound Tcf1/Lef1 to facilitate recruitment of β-catenin to target gene enhancers. Sp5 is itself directly activated by Wnt signals, thus Sp5 is proposed to be a Wnt/β-catenin pathway-specific transcription factor that functions in a feed-forward loop to robustly activate select Wnt target genes [21].

***Stem Cells*:** Yi Arial Zeng (Shanghai Institutes for Biological Sciences, China) presented work identifying the multipotent mammary stem cell using the novel Wnt target gene *Procr* (protein C receptor). The mammary gland is composed of multiple types of epithelial cells which were presumed to be generated by mammary stem cells (MaSCs) residing at the top of the hierarchy. Here her group demonstrated that Procr specifically marks the multipotent MaSC; the $Procr^{+}$ cells display high regenerative capacity in transplantation assays and differentiate into all lineages of the mammary epithelium by lineage tracing. These multipotent MaSCs exhibit epithelial-to-mesenchymal transition characteristics, and express low levels of basal keratins [22].

Toby Phesse presented work from the Vincan laboratory (University of Melbourne, Australia) that demonstrates Frizzled7 functions as a Wnt receptor in $Lgr5^+$ gastrointestinal stem cells. Intestinal epithelium regeneration *in vivo* and organoid formation *in vitro* were impaired in the absence of Frizzled7 [23]. Several other talks also focused on Frizzled7 in stem cells. Karl Willert (University of California, San Diego, USA) reported pluripotency in human embryonic stem cells is maintained by Frizzled7 [24]. He also demonstrated that the level of Wnt signalling is critical in maintaining stem cell pluripotency. Using fibroblasts from patients with a rare genetic syndrome called Focal Dermal Hypoplasia (FDK), which is caused by mutations in the *PORCN* (codes for Porcupine) gene he provided genetic evidence that WNT signalling is required to induce and maintain the pluripotent stem cell state. FDH fibroblasts are recalcitrant to standard reprogramming protocols. This blockade to reprogramming is overcome by the ectopic activation of the WNT/β signalling pathway, demonstrating that WNT signalling is an essential regulator of the induction of pluripotency. Furthermore, he found that these FDK fibroblasts are exquisitely sensitive to the level of ectopic WNT signalling: low levels of WNT signalling are required to promote reprogramming and maintain the pluripotent state whereas high levels of WNT signalling drive chromosomal instability. These results underscore the importance of WNT signalling to cellular reprogramming, a finding that is relevant to regenerative processes. Simultaneously, these findings highlight the precarious nature of ectopic WNT activation, and its tight relationship with chromosomal instability and oncogenic transformation [25].

Nick Barker (A-STAR Institute of medical Biology, Singapore) reported the identification of stem cells of the ovarian epithelium. The ovary surface epithelium (OSE) undergoes ovulatory tear-and-remodelling throughout life. Resident stem cells drive such tissue homeostasis in many adult epithelia, but their existence in the ovary was yet to be definitively proven. Lgr5 marks stem cells in multiple epithelia. He used reporter mice and Single Molecule Florescent-in-Situ-Hybridization (FISH) to document candidate $Lgr5^+$ stem cells within the mouse ovary and associated structures. Lgr5 is broadly expressed during ovary organogenesis, but becomes limited to the OSE in early neonate life. In adults, Lgr5 expression is predominantly restricted to proliferative regions of the OSE and the fimbria-mesovarian junction. Using conditional *in vivo* lineage tracing he demonstrated that embryonic and early neonate $Lgr5^+$ populations function as stem/progenitor and cells contribute to the development of adult OSE and granulosa cell lineages, as well as the epithelia of the mesovarian and oviduct, including its distal opening, the fimbria. Long-term lineage tracing reveals that adult OSE-resident $Lgr5^+$ populations contribute to epithelial homeostasis and OSE regenerative repair *in vivo*. Thus, Lgr5 is a marker of stem/progenitor cells of the ovary and tubal epithelia [26].

***Cancer/disease*:** Research from Ruby Huang and John Paul Thiery laboratories (Cancer Science Institute of Singapore) showed Frizzled7 drives an aggressive subtype (Stem-A) of ovarian cancer with stem cell properties. Knock-down of Frizzled7 induced changes which suggested the involvement of the non-canonical Wnt/planar cell polarity (PCP) pathway. Selected PCP pathway genes were found to be more highly expressed in Stem-A than non-Stem-A subgroup of ovarian cancer [27]. Philippe Merle (Hepatology Unit and Cancer Research Centre of Lyon, France) also focused on Frizzled7 in cancer, summarising the role of Wnt signalling in liver cancer [28] and provided further evidence that Frizzled7 might be a pertinent biomarker and molecular therapeutic target in liver cancer.

David Virshup's group (DUKE-NUS Graduate medical School, Singapore) and colleagues described novel inhibitors of porcupine (PORCN), an O-acyltransferase that palmitoylates Wnt, a necessary step for the secretion and biological activity of all human Wnts. They developed a novel pharmacophore whose derivatives are effective inhibitors of Wnt signalling at nanomolar concentrations. Using several experimental approaches, it was demonstrated that these compounds potently inhibit PORCN catalytic activity and hence suppress downstream Wnt activated signalling pathways. The inhibitory activity is stereospecific, as an (R) enantiomer is inactive. These novel inhibitors of PORCN are orally bioavailable and are highly efficacious in reversing tumour growth in MMTV-WNT1 mice and in tumour

xenografts derived from teratocarcinoma and pancreatic cell lines. Treated tumours show marked nuclear exclusion and decreased cytoplasmic staining of β-catenin compared to vehicle controls. Importantly the treatment modulates downstream markers of Wnt signalling and promotes differentiation of pancreatic tumours. In addition to being efficacious for the treatment of Wnt driven cancers, his data also shows that blockade of Wnt secretion with PORCN inhibitors dramatically attenuates kidney fibrosis in a mouse model of unilateral ureteral obstruction by preventing Wnt secretion from its diverse cellular sources that includes circulating inflammatory cells and intrinsic kidney cells. The expression of collagen is significantly reduced in the obstructed kidneys from the C59 treated. There is also reduced expression of Wnt target genes and nuclear exclusion of β-catenin in the treated kidneys compared to the vehicle controls. No signs of toxicity are observed in mice treated with porcupine inhibitors at therapeutically effective doses. These results on C59 demonstrate that inhibiting the Wnt/β-catenin pathway by targeting PORCN with small-molecule inhibitors is a feasible and nontoxic strategy. Use of porcupine inhibitors overcomes the problem of Wnt gene redundancy, thereby, providing new options for therapy in diseases with high Wnt activity.

Ciara Metcalfe (Fred de Sauvage laboratory, Genentech, California, USA) presented work on the role of $Lgr5^+$ intestinal stem cells in homeostasis and regeneration. Using the Lgr5DTR mouse model, it was shown that intestinal homeostasis can be maintained, at least in the short term, following the ablation of the $Lgr5^+$ stem cell population. In contrast though, ablating $Lgr5^+$ cells in the context of radiation-induced intestinal damage causes acute, catastrophic crypt loss and deterioration of crypt-villus architecture; suggesting that $Lgr5^+$ stem cells may have features that distinguish them from alternative stem cell pools. She detailed the consequences of depleting $Lgr5^+$ stem cells in xenografts models derived from a specific sub-set of colorectal cancer patients, using a novel candidate therapeutic antibody. An approach that holds significant promise for a sub-set of colorectal cancer patients [29].

Kathryn Davidson (University of Melbourne, formerly from the Moon laboratory, University of Washington, Seattle) stepped in to present Randall Moon's plenary talk summarizing Wnt regulation in recurrent and metastatic melanoma [30]. Modern therapies of metastatic melanoma, such as treatment with inhibitors of mutant BRAF, have done little to increase patient survival, in part because tumours become resistant to these inhibitors. In contrast to colorectal cancer, metastatic melanoma with elevated nuclear β-catenin is associated with better prognosis in patients who were naïve to treatment with BRAF inhibitors. Furthermore, Wnt/β-catenin signalling is required for apoptosis induced by BRAF inhibitors in metastatic melanoma cells. This talk also reviewed data supporting the idea that WNT5A-mediated signalling promotes the growth of metastatic melanoma cells by activating the phosphatydil inositol 3 kinase (PI3K)/AKT pathway in a β-catenin-independent manner. PI3K/AKT signalling is increased in patients who developed resistance to BRAF inhibitors. Intriguingly, WNT5A/AKT signalling is required for the growth of melanoma cells which are resistant to BRAF inhibitors. The evidence collectively supports the idea that WNT5A signalling may be a promising target for melanoma therapies.
The other plenary presentation of the meeting was by Tony Burgess (Walter and Eliza Hall Institute, Melbourne, Australia) who summarized our current understanding of the role of the Wnt pathway in colon cell biology, cryptogenesis and cancer. He emphasized the need for a Systems approach to colon crypt biology, and the progress in developing a Systems approach which will predict the behaviour of normal, adenoma and cancerous colon cells and crypts to changes to the growth factor/cytokine network. The strong link between Wnt/APC/β-catenin signalling and colon cancer biology (>80% of colon cancers have mutations in this pathway) suggest a strategy for killing adenoma and/or colon cancer cells which targets Wnt signalling.

Conclusions:
The Wnt meetings provide a platform to discuss cutting edge research and the Australian EMBO workshop kept up this culture of presenting outstanding research before publication. After the final

session of the meeting, several prizes were awarded. Early career prizes were awarded to Matthias Zebisch, Tor Espen Thorvaldsen and Tao-Hsin Chang, while the "Bienz-Waterman" prize for best early/mid-career female presenter was awarded jointly to Arial Zeng and Ciara Metcalf. The "Bienz-Waterman" prize (coined the "Marian" prize) honours the outstanding contribution of Mariann Bienz and Marian Waterman to the Wnt field.

In memory of our dear friend and colleague Alan Clarke (1963-2015).

"Wnters" down-under: Inaugural Australian EMBO workshop, Cable Beach, Broome, WA, 2014

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