Signalling through the grapevine

Ivan Dikic & Roger J. Daly

The 5th Barossa Meeting on 'Cell Signalling and Molecular Medicine' was held in November 2011 in the Barossa Valley, South Australia. The combination of an inspirational environment and outstanding science led to a superb meeting that highlighted the versatility of cellular signalling systems and how they can be targeted by novel therapeutic approaches.

Given that the famous French scientist Louis Pasteur once proclaimed that "A bottle of wine contains more philosophy than all the books in the world", Angel Lopez, Stuart Pitson and Greg Goodall (Centre for Cancer Biology, Adelaide, Australia), convenors of the 5th Barossa Meeting on Cell Signalling and Molecular Medicine, were probably quietly confident that their conference, held among the rolling hills of the Barossa Ranges, overlooking the vineyards of one of Australia's premier winemaking areas, would successfully promote thought, discussion and collaborative interaction. Their confidence was justified, since the combination of outstanding science, stunning location and superb food and wine led to a meeting that was stimulating to both the intellect and the senses. Perhaps the only downside is that wine-tasting events at other conferences will now pale in comparison, but at least a new benchmark has been set!

The first major theme that emanated from the meeting was the remarkable versatility of particular signalling systems and molecules, which reflects the evolution of a plethora of protein–protein interaction domains that 'decode' particular signals with high specificity, as well as integration and spatiotemporal control of signalling events within the cell. This was highlighted by a series of talks focusing on the ubiquitin signalling system. The past decade has seen major developments in our understanding of how modification of proteins by the addition of ubiquitin monomers or chains regulates diverse biological responses. In particular, it is evident that chain assembly involving different linkages—for example, Lys48, Lys63 or the amino-terminal methionine of ubiquitin linked to the carboxy-terminal glycine of

the adjacent molecule (the latter often referred to as a linear linkage)—generates specific signals that are 'decoded' by binding to distinct ubiquitin-binding domains (UBDs) present on other signalling proteins [1]. A prime example of a receptor signalling system that uses specific ubiquitination events to regulate complex cellular responses is the tumour necrosis factor receptor 1 (TNFR1). Here, Lys63- and Lys11-linked ubiquitin chains generated on receptor-interacting kinase 1 (RIP1) by TNFR-associated factor 2 (TRAF2) and associated cellular inhibitor of apoptosis proteins (cIAPs) 1 and 2, recruit TAK-binding protein/transforming growth factor- β -activated kinase (TAB/TAK), leading to transcriptional activation of NF- κ B and AP1 through inhibitor of κ B kinases (IKKs) and mitogen-activated protein kinases (MAPKs),

respectively. In addition, the linear ubiquitin chain assembly complex (LUBAC), comprising HOIL1 and HOIP, is recruited to the TNFR1 where it is required for ubiquitination of NF- κ B-essential modulator/inhibitor of κ B kinase- γ (NEMO/IKK) and efficient activation of NF- κ B by TNF. John Silke (Walter and Eliza Hall Institute of Medical Research, Australia) described how Smac mimetics activate the cIAP1 E3 ligase by converting cIAP from a monomeric form to a dimeric active RING form [2] and proposed that a similar mechanism occurs upon recruitment to the TNFR1 complex to activate cIAPs E3 ligase activity. Henning Walczak (Imperial College London, UK) then provided an in-depth characterization of one of the LUBAC components, SHARPIN. SHARPIN is recruited to both the CD40 and TNFR1 signalling

complexes together with HOIL1 and HOIP in a tripartite complex. *In vitro*, a SHARPIN–HOIP complex, as well as the tripartite SHARPIN–HOIP–HOIL1 and the previously characterized HOIP–HOIL1 complexes, generate linear ubiquitin chains and are able to ubiquitinate NEMO. Importantly, major insights into the physiological role of SHARPIN were provided by the phenotypic characterization of chronic proliferative dermatitis (*cpdm*) mice, which harbour a spontaneous mutation in the Sharpin gene and exhibit inflammatory skin lesions and defective lymphoid organogenesis. Primary keratinocytes isolated from this strain exhibited impaired NF- κ B activation in response to TNF, and *cpdm* fibroblasts were highly sensitive to TNF-induced cell death. Since the *cpdm* skin phenotype was rescued upon crossing with TNF-deficient mice, this leads to a model in which the absence of SHARPIN causes enhanced keratinocyte cell death, that in turn promotes the inflammatory phenotype [3].

...the remarkable versatility of particular signalling systems and molecules [...] reflects the evolution of a plethora of protein–protein interaction domains that ‘decode’ particular signals with high specificity

Ivan Dikic (Goethe U. Medical School, Germany) presented structural data explaining the specificity of NEMO binding to linear ubiquitin chains. NEMO contains UBAN (the ubiquitin-binding domain in ABIN and NEMO proteins), which is a dimer of the helical fold that binds with high selectivity to linear ubiquitin [4]. It is thought that LUBAC-mediated NEMO ubiquitination promotes *trans*-binding of neighbouring NEMO molecules leading to conformational changes and activation of associated IKK α and IKK β . An additional level of complexity to this receptor signalling system was described by Sarah Spiegel (Virginia Commonwealth U., USA), who presented data indicating that sphingosine 1-phosphate (S1P) binds to the N-terminal RING domain of TRAF2 and stimulates its ability to generate Lys63-linked ubiquitin chains on RIP1 [5]. This clarifies the role of sphingosine kinase and S1P in canonical NF- κ B signalling and provides a mechanistic explanation for the

...TBK1 mediates phosphorylation of OPTN and p62 in order to mediate the specificity in binding of their UBDs, causing efficient recognition of ubiquitinated *Salmonella*

observed effects of SphK1 on biological end points such as inflammation and cell survival. Overall these data highlight how modulating the levels of a particular type of ubiquitin chain can modulate signal output and cellular responses.

Two talks described novel roles of the ubiquitin signalling system in regulating cellular responses to bacterial or viral infection. Ivan Dikic presented studies that characterized how phosphorylation of the autophagy receptor optineurin (OPTN) determines the balance between autophagic clearance of *Salmonella enterica* and proliferation of this bacterium in the cytosol. OPTN binds to ubiquitin-coated bacteria via its UBAN domain and to autophagy modifiers present on autophagosomal membranes via an LC3-interacting motif (LIR). Interestingly, Tank-binding kinase (TBK1) phosphorylates OPTN on Ser177 adjacent to the LIR, which increases binding affinity towards the autophagy modifier LC3B and hence autophagic clearance [6]. Dikic presented new data to demonstrate that TBK1 mediates phosphorylation of OPTN and p62 in order to mediate the specificity in binding of their UBDs, causing efficient recognition of ubiquitinated *Salmonella*, as well as subsequent recruitment of autophagosomal membranes. These insights highlight the interplay between phosphorylation- and ubiquitin-based signalling systems. Given that several other autophagy receptors also contain conserved serine residues adjacent to their LIRs, the regulation of autophagy modifier–receptor interaction by phosphorylation might be a more broadly applicable mechanism than previously thought. Zhijian Chen (U. Texas Southwestern Medical Center, USA) described the use of a cell-free system to study how the RNA sensor RIG1, which detects viral infection, signals to the transcription factors NF- κ B and IRF3. This led to the discovery that RIG1 activation involves binding of both RNA and unanchored Lys63-linked polyubiquitin chains to the RIG1 tandem CARD domains. In addition, the CARD domains of RIG1 bind to the CARD domains of the

mitochondrial adaptor protein MAVS, leading to its aggregation and IRF3 activation. Interestingly, recombinant MAVS protein forms fibrous polymers that exhibit prion-like activity in causing aggregation of endogenous MAVS [7].

Tony Pawson (Samuel Lunenfeld Research Institute, Canada) highlighted the remarkable versatility of the adaptor protein Shc1, previously termed ShcA. Earlier studies had demonstrated that Shc1 regulates diverse biological processes, hinting that it might be embedded within a complex but ill-defined signalling network. In order to define this network, the Pawson group used mass spectrometry to identify multiple EGF-induced phosphorylation sites on Shc1 and approximately 40 proteins that associate with it upon EGF stimulation. They then established a scheduled multiple reaction monitoring (sMRM) workflow that enabled accurate quantification of Shc1 phosphosites and interactors over space and time. This revealed that Shc1 is subject to distinct waves of phosphorylation and protein association–dissociation events in response to EGF, with early tyrosine phosphorylation followed by later serine/threonine phosphorylation, and initial Grb2-dependent recruitment of proteins that mediate mitogenic signalling followed by later Grb2-independent binding of proteins involved in cytoskeletal reorganization. These data indicate that signalling by Shc1 is far more complex than originally suspected and provide important mechanistic insights into the pleiotropic role of Shc1 within the cell. Interestingly, Roger Daly (Garvan Institute of Medical Research, Australia) reported that one of the novel Shc1 interactors identified by the Pawson group, the atypical kinase SgK269, is a key component of an Src family kinase signalling network that characterizes basal breast cancer cells. Overexpression of SgK269 in MCF-10A mammary epithelial cells promotes growth in three-dimensional culture and perturbs acinar morphogenesis, indicating that SgK269 might contribute to progression of this aggressive breast cancer subtype.

...Shc1 is subject to distinct waves of phosphorylation [...] in response to EGF, with early tyrosine phosphorylation followed by later serine/threonine phosphorylation

The second theme that emerged from the meeting was that multiple levels of cellular signalling, from cell surface receptors to microRNAs, can be targeted by therapeutic approaches. Two talks focused on granulocyte-macrophage colony-stimulating factor (GM-CSF) signalling, which is relevant to pathologies such as rheumatoid arthritis and leukaemia. Michael Parker (St Vincent's Institute of Medical Research, Australia) and Timothy Hercus (Centre for Cancer Biology, Adelaide, Australia) presented new X-ray crystallographic data regarding the structure of the binary complex formed between GM-CSF and GMR α , the ligand-specific α -subunit of the GM-CSF receptor signalling system. Together with mutagenesis studies on GMR α , these studies provide important new information regarding the GM-CSF binding surface and mode of receptor complex assembly that opens avenues for therapeutic intervention with antibodies or small molecule drugs. In addition, John Schrader (The Biomedical Research Centre, Vancouver, Canada) described the isolation and characterization of human monoclonal autoantibodies against GM-CSF from patients with idiopathic pulmonary alveolar proteinosis. Since the majority of these antibodies neutralized the bioactivity of GM-CSF, they offer significant therapeutic potential.

While cytokines and their receptors represent well-established therapeutic targets, two talks emphasized how the recent renaissance of interest in RNA biology and signalling has led to the identification of novel targets and strategies for therapeutic intervention. Frank Slack (Yale U., USA) described the establishment of a pre-clinical model to study the therapeutic targeting of a particular microRNA that is upregulated in certain haematological malignancies. Slack's group generated a transgenic mouse model that exhibits Cre-regulated and inducible expression of this microRNA, and splenic expression led to the development of a precursor B-cell lymphoblastic lymphoma/leukaemia. Importantly, local or systemic delivery of nanoparticles harbouring a targeted anti-microRNA led to tumour regression characterized by increased cell apoptosis. It will be interesting to determine the efficacy of this approach against xenografts generated from human cancers that contain other oncogenic aberrations in addition to dysregulation of this microRNA. In the second

talk, Ross Hannan (Peter MacCallum Cancer Centre, Australia) took the concept of non-canonical drug targets to the extreme by 'drugging the undruggable', specifically, ribosomal RNA gene (rDNA) transcription. His group has tested the hypothesis that Myc-driven cancers exhibit increased sensitivity to inhibition of RNA polymerase I (Pol I)-driven rDNA transcription and hence ribosome biogenesis. This was done by using a small molecule, CX5461, which targets the Pol I transcription initiation factor complex [8]. Treatment with the drug delayed the onset of transplanted E μ -Myc lymphomas in mice in a manner dependent on the presence of wild-type p53. Induction of apoptosis by the drug reflects a p53-dependent nucleolar stress response, rather than ribosome deficiency. Importantly, CX5461 selectively kills malignant B cells and does not activate p53 in normal B cells. In the light of these exciting data, CX5461 will enter human clinical trials in 2012.

...multiple levels of cellular signalling, from cell surface receptors to microRNAs can be targeted by therapeutic approaches

An alternative strategy for tackling seemingly 'undruggable' targets is to think outside the box, and this approach was highlighted by Paul Neilsen (U. Adelaide, Australia), who described his group's work in developing therapeutic approaches that are selective for cancers expressing mutant p53. By using H1299 lung cancer cells engineered to allow the induced expression of various p53 mutants in a p53-null background, in combination with expression profiling and ChIP analyses, Neilsen demonstrated that mutant p53 forms a complex with p63 at the promoters of p63 target genes, which disrupts normal p63 function and induces several novel targets including miR-155 and PLK2. Importantly, mutant p53-expressing cells were more sensitive to the small molecule PLK2 inhibitor BI2536, identifying a potential targeted therapy for cancers exhibiting p53 mutations.

It was fitting that the 2011 Clifford Prize recipient, Vishva Dixit (Genentech, USA), gave the final talk at the meeting and highlighted an emerging class of drug target. The

Clifford Prize is tightly linked to the Barossa symposia series and recognizes excellence in cancer research that has laid the foundation for novel and significant therapies. Dixit was a popular and worthy winner owing to his seminal research characterizing the death-receptor-induced apoptotic pathway and the inflammasome complex. His talk addressed an intriguing question: how do human cancers overexpress proteins that are normally turned over rapidly? The proteins Dixit chose for study were the inhibitors of DNA binding (Id)1–3, which antagonize specific basic helix–loop–helix transcription factors and thereby promote 'stemness'. Interestingly, USP1 acts as a de-ubiquitinating enzyme (DUB) for the Ids and thereby attenuates their proteasomal degradation. Furthermore, USP1 and Id2 are both overexpressed in osteosarcoma, and USP1 knockdown reduces growth of osteosarcomas in a mouse model and, surprisingly, induces differentiation to bone [9]. This talk highlighted USP1 as a potential target for differentiation therapy, which is of great interest given the 'druggability' of DUBs and emerging drug development activity in this area.

The next Barossa Meeting on Cell Signalling is not until November 2013, and we are sure that huge advances will be made on these and other related topics between now and then.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- Ikeda F, Crossetto N, Dikic I (2010) *Cell* **143**: 677–681
- Feltham R *et al* (2011) *J Biol Chem* **286**: 17015–17028
- Gerlach B *et al* (2011) *Nature* **471**: 591–596
- Rahighi S *et al* (2009) *Cell* **136**: 1098–1109
- Alvarez SE *et al* (2010) *Nature* **465**: 1084–1088
- Wild P *et al* (2011) *Science* **333**: 228–233
- Hou F *et al* (2011) *Cell* **146**: 448–461
- Drygin D *et al* (2011) *Cancer Res* **71**: 1418–1430
- Williams SA *et al* (2011) *Cell* **146**: 918–930

Ivan Dikic is at the Goethe University Medical School, Frankfurt, Germany. Roger Daly is at the Cancer Research Program, Garvan Institute of Medical Research, Sydney, Australia and St Vincent's Hospital Clinical School, Department of Medicine, University of New South Wales, Australia. E-mail: r.daly@garvan.org.au

EMBO reports (2012) **13**, 178–180; published online 21 February 2012; doi:10.1038/embo.2012.16