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SPATA2 – Keeping the TNF signal short and sweet

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In this issue of *The EMBO Journal*, a multi-layered global proteome resource, which comprehensively covers TNF-induced phosphorylation and ubiquitination events as well as receptor interactions, has led to identification of SPATA2 as a novel player that contributes to the TNF signalling by interacting with the LUBAC ubiquitin ligase and the CYLD deubiquitylase. Loss of SPATA2 augments transcriptional activation of NF-κB and limits TNF-induced necroptosis. A separate screen published in *EMBO Reports* corroborates these findings by showing that SPATA2 deficiency regulates CYLD activity, TNF-induced NF-κB and cell death.

See also: **SA Wagner *et al* (June 2016)**; **L Schlicher *et al* *(July 2016)***

The Spartans, famed for their brevity of speech, gave rise to the adjective laconic, meaning of few words, but literally, from Laconia, the kingdom of Sparta. For example, when asked by Philip of Macedon, who was threatening the Spartans with invasion, whether they wished that he should come as a "friend" or a "foe"; they replied, “Neither”. While it is impossible to match Spartan wit, Wagner *et al* (2016) have employed an impressive proteome wide quantitative mass spectrometry (MS) approach to dissect the molecular cascade of phosphorylation and ubiquitylation events that follow stimulation with TNF, one of the most studied signalling pathways in biology. Surprisingly, in addition to revealing a series of unknown post-translational modifications, which we discuss below, this analysis yielded identification of a novel player in the TNF signalling pathway, Spermatogenesis Associated 2 (SPATA2), which, like its namesake, helps maintain the brevity of TNF signalling. SPATA2 interacts with the LUBAC component HOIP and the deubiquitylase (DUB) CYLD, and is required for the recruitment of CYLD to the TNF signalling complex. SPATA2 was also identified as a CYLD interacting protein in a separate screen by Schlicher *et al* (2016) and both groups show that loss of SPATA2 augments TNF induced transcription and limits TNF-induced cell death.

Wagner *et al* used triplex SILAC labeling (allowing simultaneous measurement of un-liganded, 5 and 15 min TNF stimulation states) in combination with TiO_2-based enrichment for phosphorylated peptides, di-Gly immunoprecipitation for ubiquitylated peptides and Flag-TNF affinity purification. Tryptically digested samples were analyzed with nanoHPLC coupled to a Q-Exactive mass spectrometer. Globally, this analysis identified almost 9,000 phosphorylated peptides and fractionally less modified with ubiquitin. About 8% of proteins had increased phosphorylation status upon TNF stimulation while about 1% of the ubiquitylated proteins identified became more modified.

The authors were also able to monitor the temporal association of proteins with the TNF receptor signalling complex (TNF-RSC). Satisfyingly they identified many known signalling components such as TRAF2, RIPK1, cIAP1 and LUBAC (the heterodimeric complex comprising SHARPIN, HOIL and HOIP). They were also able to quantify the dynamic association of proteins with the TNF-RSC, together with specific phosphorylation and ubiquitylation events. Like presents on Christmas day many of the findings will bear more detailed study at a later time, but some of the shiny new findings are uncharacterized phosphorylation sites, in the context of TNF signalling, such as NFκB2 (S870, S23), HOIP (S383), Sharpin (S165), TBK1 (S172) and CYLD (S418). Some of these phosphorylation events, and the fact that TBK1 was recruited to the TNF-RSC, suggest that TNF can activate the non-canonical NF-κB pathway. To date this has only been shown in TNF stimulated cells using dominant negative NIK mutants and RIPK1 knock-out cells, but not as a normal physiological response (Kim et al, 2011; Gentle et al, 2011; Malinin et al, 1997). Likewise, there are uncharacterized ubiquitylation sites, including HOIP (K330), Sharpin (K189, K154) NEMO (K285), TNFR1 (K347) and MLKL (K95). Interestingly, in contrast to the rapid ubiquitylation of most other TNF-RSC components, modification of TNFR1 at K347 peaked after 15 minutes of stimulation. This delayed modification suggests that this site is important for the regulation of the signalling complex at later stages, and it is an enticing speculation that this site may be the undetermined signal that transitions complex-I to complex-II.

Leaving these tantalising insights aside for future work, the authors focused on deciphering the function of SPATA2. cDNA from Spermatogenesis-associated protein 2 (SPATA2) was originally isolated from human testis in 1999. It was shown to be expressed at the protein level in testes and to a lesser extent in spleen, thymus and prostate and may play a role in spermatogenesis (Graziotto et al, 1999; Onisto et al, 2000). The 60 kDa protein contains an N-terminal PUB (PNGase/UBA) domain, akin to that found in HOIP, and a C-terminal PHD finger. By performing a second round of MS using SPATA2 as the bait protein, Wagner *et al* (2016) demonstrated that SPATA2 interacted with CYLD as well as all three components of LUBAC, in unstimulated conditions. And as already mentioned Schlicher *et al* (2016) identified SPATA2 as a CYLD interacting partner. Co-immunoprecipitation experiments verified that these interactions occur in steady state conditions similar to the interaction between OTULIN and HOIP and CYLD and HOIP. Both HOIP and SPATA2 were capable of co-immunoprecipitating CYLD whereas OTULIN was only detected in HOIP pull downs, indicating that CYLD and OTULIN interact mutually exclusively with HOIP. The N-terminal PUB domain-containing portion of SPATA2 is sufficient for the interaction with CYLD whereas the C-terminal PHD finger-containing portion interacts with HOIP. The PUB domain-containing portion of SPATA2 interacts with the catalytic USP domain of CYLD making it possible that SPATA2 influences the DUB activity of CYLD. Indeed, Schlicher *et al* (2016) showed using *in vitro* ubiquitin cleavage assays that purified CYLD had more deubiquitylase activity against K63 and M1, but not K48, linked ubiquitin chains when co-expressed with SPATA2. The connection with CYLD is interesting because CYLD has previously been associated with the regulation of spermatogenesis. Genetic deficiency of CYLD results in reduced testes size, attenuated germ cell apoptosis and impaired spermatogenesis leading to sterility in male mice (Wright et al, 2007), thus a SPATA2-CYLD complex may regulate spermatogenesis.

Both groups showed that SPATA2 is recruited to the TNF-RSC within 5 minutes of TNF stimulation along with other components such as TNFR1, TRADD, RIPK1 and CYLD. While SPATA2 is required for the recruitment of CYLD to the TNF-RSC, surprisingly, in the absence of SPATA2, Wagner *et al* (2016) detected less RIPK1 ubiquitylation. Consistent with the expected effects of loss of a DUB it has been shown that cells lacking CYLD show a marked increase in the amount of ubiquitylated

RIPK1 upon TNF treatment (Moquin et al, 2013; Draber et al, 2015). Hence, there is a significant difference between removing CYLD compared with impairing its ability to be recruited to the TNF-RSC. Because the cIAPs and LUBAC are well-characterised E3 ligases for RIPK1, it will be interesting to find out whether they are recruited to the TNF-RSC in the absence of SPATA2. With regard to LUBAC, silencing of SPATA2 limited the recruitment of Sharpin to the TNF-RSC although, surprisingly given the trimeric nature of the complex, HOIP recruitment was seemingly unaffected. Mutation of Sharpin affects LUBAC activity (Gerlach et al, 2011) so it is unclear how this asymmetric recruitment might affect LUBAC activity. It is also possible that the apparent reduction in the RIPK1 ubiquitylation smear in the absence of SPATA2 actually represents a qualitative difference in the ubiquitin chain linkages rather than a reduction.

Given that SPATA2 is required to link CYLD to the TNF-RSC and that CYLD is an important negative regulator of TNF-induced NF-κB, it was unremarkable that loss of SPATA2 increased the TNF-induced NF-κB response. However, Wagner *et al* (2016) did not observe any changes in IκBα degradation or the phosphorylation status of p65 or p38 upon loss of SPATA2. This contrasts with Schlicher *et al* (2016) who showed dramatic acceleration of TNF induced IκBα degradation, p65, p38, JNK and ERK phosphorylation and prolongation of these events in two different SPATA2 depleted cell lines when compared with their normal counterparts. Like Wagner *et al* (2016), Schlicher *et al* (2016) showed that depletion of SPATA2 helped protect cells from TNF-induced cell death. Surprisingly this protection occurred even in TAK1 deficient cells (Schlicher *et al* (2016)). Because loss of TAK1 prevents activation of NF-κB this clearly shows that loss of SPATA2 can protect cells in an NF-κB independent manner. Consistent with this they showed that RIPK1 recruitment into complex 2, as determined by FADD pull down, was reduced in cells doubly deficient in TAK1 and SPATA2. This needs to be explored further because they were unable to analyse caspase-8 recruitment into complex 2, however, combined with the lack of cell death, the result suggests that loss of SPATA2 impairs the formation of caspase-8 containing complex 2.

These new results make a significant contribution to the TNF signalling field and because LUBAC is involved in other NF-κB activating signalling pathways, such as

NOD and TLR, it is likely these too will be regulated by SPATA2. Furthermore the study by Wagner and colleagues provide an important and rich resource for future studies to investigate the function of post translational modifications in TNF signalling and disease. Protein phosphorylation and ubiquitylation are integral to TNF signalling and this study highlights how little we know about site-specific modification of many of the well-characterised TNF-RSC components. While the identification of SPATA2 is exciting, it is clear that much remains to completely define its role. It will therefore be interesting to extend these observations *in vivo* and to other death receptor signalling pathways, or situations where LUBAC and CYLD are essential.

A picture is worth a thousand words, but even our, too complex, figure captures only a fraction of the information generated by Wagner and colleagues. New ways of visualising such intricate data are therefore clearly needed. It seems fitting to bear the Spartan ideal in mind when thinking about such new approaches. Since their responses were clearly intended to be the final word; it is also fitting to leave the last word to them: following a long winded ambassadorial address, the Spartans responded that since they could no longer remember the first half of the speech they could make nothing of the second. The ambassadors came back the next day with a three-word request, which was granted. It would have been amusing to see how the Spartans could have described the wealth of data generated by Wagner *et al*: TNF signalling needs more work?

Figure 1 A complex schematic overview of the TNF signalling pathway. The left hand side indicates many (but not all) of the known interactions, modifications and outcomes following binding of TNF to its receptor and includes the new player identified by Wagner *et al.* (2016), SPATA2 (red). On the right hand side are novel, in the context of TNF signalling, modifications resulting from TNF signalling discovered by the same group. In addition to modifications highlighted in the paper, we have selected some additional ones based on the fact that they are high at 5 & 15 minutes, in both repeat experiments and low in the crapome database (Mellacheruvu et al, 2013).

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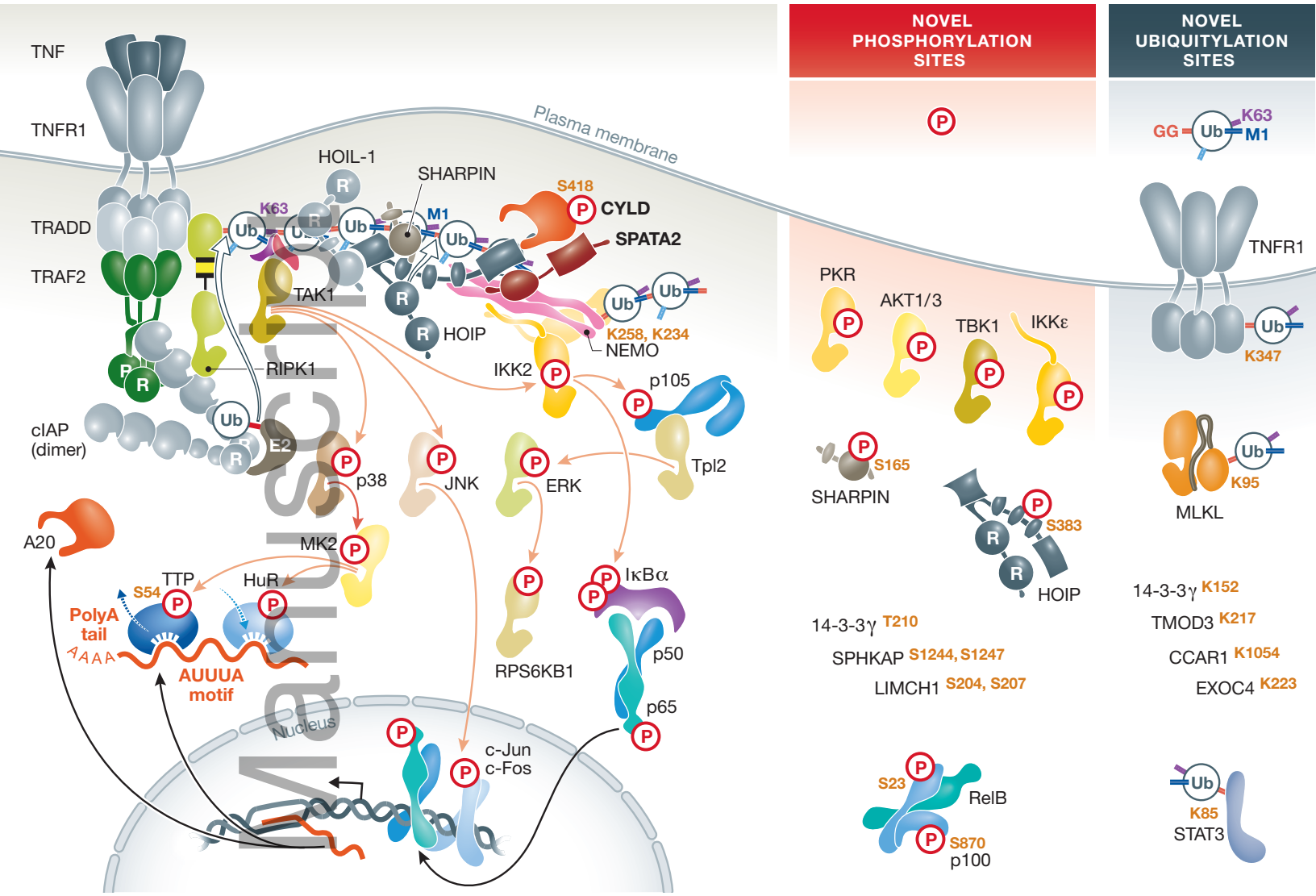

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