# Exploring the nature of ubiquitin linkage-specific binding: from ubiquitin binding domains to full-length proteins

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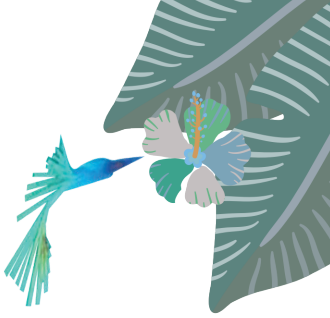

# 6

# Discussion

## UBIQUITIN LINKAGE-SPECIFIC BINDING

In the elaborate world of proteins, ubiquitin is only a tiny protein; counting no more than 76 amino acids. Despite its small size however, it can regulate a wide range of cellular processes, as discussed in **chapter 1**. Part of this ability to influence so many processes comes from its capacity to form ubiquitin polymers through 8 internal, different conjugation sites. Several proteins can recognize one or a few of the polyubiquitin variants that result from this self-conjugation: a phenomenon called linkage-specific binding. As can be imagined, a complex world originates from the plethora of possible polyubiquitin variants; one that is still not fully understood due to lack of proper tools.

This thesis describes an effort to create a landscape of ubiquitin linkage-specific binding through various approaches. The journey starts from small, isolated ubiquitin binding domains (**chapter 2**) and proceeds to finding linkage specific ubiquitin binding proteins using non-hydrolyzable 'click' diubiquitin proteins as bait (**chapter 3** and **4**). From there, **chapter 5** of this thesis characterizes the functional consequences of one of the major hits found: $^{K27}Ub_2$ binding to the deubiquitinase UCHL3.

Ubiquitin linkage-specificity and the recognition thereof is an important concept in the tight regulation of cellular activity. Although the screening methods presented here provide a new foundation for understanding how ubiquitin linkage types can influence so many different cellular processes, much more can be learned if we extend the scope further, as will be discussed in the final paragraph of this chapter.

## SCREENING UBIQUITIN BINDING DOMAINS FOR UBIQUITIN LINKAGE-SPECIFIC BINDING

When we distill ubiquitin linkage-specific binding capacity to the most basic elements, we must focus on ubiquitin binding domains (UBDs) in particular. UBDs are small fragments found in proteins that usually consist of a typical structural fold and a certain sequential consensus motif. Several UBDs have been reported so far that have a preference for a certain ubiquitin linkage-type (Husnjak and Dikic, 2012), but have in the past been more restricted to the typical K48-, K63 and M1-ubiquitin type. In **chapter 2**, we investigated a FP-based method to screen synthesized, α-helical ubiquitin binding domains for binding to all of the 8 different homotypical linkages possible. Most strikingly, we found a set of UBDs that bound to $^{K6}Ub_2$ specifically. We then characterized the $^{K6}Ub_2$ binding site on the two most potent hits and revealed a shared binding signature.

### Zinc-finger UBDs and the SPR-screen

The FP screen however did not allow for measurement of UBDs from the zinc-finger class (ZnF) type since their folding could not be guaranteed. In addition to a synthetic approach, we therefore established an expression-based system where we designed an *E. coli* vector with a N-terminal GST affinity purification tag and a C-terminal biotinylation tag (AviTag, Avidity LLC). Using this system, we screened an additional 129 ZnF domains. Although the results of this screen are not discussed in detail in this thesis, some interesting leads were

found. Table 1 therefore describes some promising candidates from the SPR screen, with the most potent or interesting ones highlighted bold. It must be noted that none were validated with orthogonal techniques however, except for the NZF domain of YAF2. Using our FRET diubiquitin pairs (Geurink *et al.*, 2016), we confirmed NZF YAF2 interaction with $^{K33}Ub_2$ and potentially $^{K29}Ub_2$. Using biolayer interferometry, we could confirm that the NZF domain of YAF2 indeed binds to K29- and K33-linked diubiquitin, although no $K_D$ values could be determined. YAF2 is an interesting candidate to investigate further, considering it is an important regulator of Myc activity (Mädge *et al.*, 2003), Polycomb repressive complex 1 (PRC1) (Rose *et al.*, 2016), PCDC5 stability (Park *et al.*, 2015) and YY1-mediated myogenic regulation (Kalenik *et al.*, 1997). Interestingly, the interaction of YAF2 with PCDC5 has been mapped partially within the designated NZF domain (Park *et al.*, 2015), which in light of the screening result implies that the interaction could be K29-/K33-ubiquitin regulated.

| | UBD | Gene name | Binding to |
|---|---|---|---|
| controls | **UIM2+3** | **ANK13B** | **K63** |
| | **UBA** | **FAF1** | **K48** |
| | **MIU1+2** | **FAM63A** | **K48** |
| | **UIM2+3** | **USP37** | **K48 + K63** |
| α-helical UBDs | **UBAN** | **ABIN1** | **M1** |
| | **UBAN** | **ABIN2** | **M1** |
| | **UBAN** | **ABIN3** | **M1** |
| | UBAL-ARI | AKIB1 | K11 + K63 |
| | MIU | CA124/SPRTN | K11 |
| | MIU4+5 | CCDC50 | K6 (pref) + K48 |
| | MIU4 | CCDC50 | K11 |
| | MIU5 | CCDC50 | K27 + K11 |
| | **UIM2+3** | **EPN1** | **K63** |
| | UIM2 | EPN1 | K11 (+ K33?) |
| | **UIM1+3** | **EPN1 (isoform 2 & 3)** | **K11** |
| | UIM1+2 | EPS15 | K11 + K63 |
| | UIM2 | EPS15 | K11 |
| | MIU2 | FAM63A | K6 + K48 |
| | UIM | HGS | K11 |
| | UBA | MARK1 | K11 + M1 |
| | MUI1 | RN168 | K11 |
| | MIU2 | RN168 | K11 (slight pref) |
| | UBA | SIK2 | K6? |
| | UBAL-CUEB2 | SMRCD | K11 |
| | **UBA** | **SNRK** | **K11 + K33** |
| | UBAL-N4BP | ZC12B | K6 + K48 |
| | UIM2 | ZFN2B / AIRAPL | K48 |

| | UBD | Gene name | Binding to |
|---|---|---|---|
| control | **NZF1** | **ZRAN1 / TRABID** | **K29** |
| ZnF UBDs | UBZ2 | CA086 (FAP20) | K11 + K29, M1? |
| | UBZ1 | CACO2 | K11 |
| | UBZ7 | EEA1 | K11 |
| | UBZ2 | KCMF1 | K11 |
| | NZF | NEIL3 | K11 + K29 |
| | UBZ8 | PCF11 | K11 |
| | A20 | RABX5 | K29 |
| | **NZF3** | **RBP2** | **K11 + K33** |
| | **NZF6** | **RBP2** | **K11 + K33** |
| | **NZF7** | **RBP2** | **K11 + K33** |
| | **NZF8** | **RBP2** | **K11 + K33** |
| | UBZ2 | RN166 | K6? |
| | **NZF2** | **RNF31** | **K29** |
| | **UBZ1+2** | **TAX1BP1** | **K11 + K29** |
| | UBZT5 | TRAD1 | K11 |
| | UBZT1 | TRAF4 | K11 |
| | UBZ6 | XPA | K11 |
| | **NZF** | **YAF2** | **K29 + K33** |
| | NZF2 | ZRAB2 | K29? |
| | NZF1 | ZRAB3 | K29? |

***Table 1 and 2. Overview of ubiquitin binding domains with potential ubiquitin linkage-specific binding.*** *Table 1 (left panel) describes putative domains belonging to the α-helical class. Table 2 (right panel) describes potential UBDs belonging to the zinc finger (ZnF) class. Shown in the upper panels (colored green) are reported binding domains with Ub linkage selectivity that display according behavior in the SPR screen.*

A major limitation of the expression-based SPR approach concerns the stability of the ZnF domains. To check the stability of the peptides during the runs, a mono ubiquitin control was measured after each diubiquitin series. Indeed, many ZnF domains displayed loss of signal towards the end of the screen and about 50% of the peptides disintegrated after 3 to 5 diubiquitin runs. Therefore, this screen tends to be more biased towards the first 5 measured diubiquitin types: M1, K29, K11, K48 and K33. The original data must therefore be consulted first and can be accessed through Gerbrand van der Heden van Noort (e-mail: gvanderheden@lumc.nl).

## Do $^{K6}Ub_2$ binding domains require more accessibility?

In the SPR screen we also included a selection of FP screening hits and all BGG-binding peptides. Interestingly, UBA UBAC2 and UBA$^{ext}$ UBXN1 both displayed strong binding to ubiquitin but also lost their evident preference for $^{K6}Ub_2$ observed in the FP screen. A similar trend was seen in preliminary experiments where we used the UBA UBAC2 domain as a tandem ubiquitin binding entity (TUBE) pulldown tool for K6-ubiquitinated substrates. TUBEs consist of ubiquitin binding domains cloned in tandem repeats, usually up to 4 times, which increases the overall affinity for ubiquitinated substrates so they may be captured and identified by mass spectrometry (Scott *et al.*, 2015). In the case of an UBA UBAC2 TUBE however, $^{K6}Ub_4$ and $^{M1}Ub_4$ were pulled down in equal amounts (data not shown). Both the pulldown and SPR technique have in common that the UBD is immobilized on a carrier surface, thereby restricting its mobility and potentially also the accessibility. Considering that UBA UBAC2 binding and $^{K6}Ub_2$ preference was validated by MST and ITC (chapter 2, Fig. 4) and the fact that UBA UBXN1 was previously reported as K6-polyubiquitin binding (Wu-Baer, Ludwig and Baer, 2010), an interesting consideration may be that K6 ubiquitin binding domains require more accessibility and flexibility in solution. This notion is supported by the finding that UBA UBAC2 binds two $^{K6}Ub_2$ entities simultaneously, which certainly requires more accessibility on the UBA part.

## Advantages and disadvantages of the FP and SPR screening methods

Taken together, each screening method clearly has its advantages and its limitations. While the main advantage of FP-based screening is its free, in-solution-based nature and the low concentration of fluorophore needed, it can become restricted by the need for a sufficient difference in size between the two interaction partners. An additional complicating factor was added in our screen by using synthesized UBDs, which added the uncertainty of proper folding.

High-throughput surface plasmon resonance is a powerful and sensitive method to screen potential drug candidates or antibody epitopes, but in the context used here was found to be prone to ligand immobilization and stability issues. This can be overcome by spotting new ligand before each new diubiquitin run, but it is much more costly and the advantage of comparing binding between different diubiquitin linkages to the same ligand will be lost. This could make interdependent comparison of the diubiquitin linkages more difficult, whilst this is one of the major aims of the screening event.

## SCREENING UBIQUITIN BINDING PROTEINS FOR UBIQUITIN LINKAGE-SPECIFIC BINDING

An effective approach to study linkage-specificity of ubiquitin binding proteins (UbP's) in a cellular and full-length protein context was set-up in **chapter 3** and **4** of this thesis and harnesses the power of chemical biology and click-chemistry in combination with cell biology and tandem mass-spectrometry (Zhang *et al.*, 2017, 2018). Copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC, often referred to as 'click chemistry') results in the generation of a triazole linkage which mimics the electrophysical charge of the native isopeptide bond well (Brik *et al.*, 2005). Previous studies have already shown that triazole-linked diubiquitin can be used effectively to interrogate UBD linkage-specific binding (Weikart, Sommer and Mootz, 2012) and to assess the mechanism of action of DUB's (Flierman *et al.*, 2016). In addition, we compared the binding behavior of three of our hits, UCHL3, NZF TAB2/3 and UBA2 hHR23A, to both native- and triazole-linked diubiquitin and found that there is no difference in preferential binding. A slight difference in $K_D$ between native and non-hydrolyzable linkages was found, but this was consistent over all samples, indicating that these differences originate in the used labeling chemistry and place of biotin attachment (random primary amine versus N-terminus, respectively). More recent studies using high-resolution NMR spectroscopy have shown that the triazole linkage also resembles the structural and dynamic characteristics of $^{K11}Ub_2$, $^{K27}Ub_2$, $^{K48}Ub_2$ and $^{K63}Ub_2$ well, further confirming that they can be used as effective surrogates for natively-linked ubiquitin dimers (Schneider *et al.*, 2019; Zhao *et al.*, 2019). In addition, the found Ub linkage-specific binding in our study was confirmed by independent studies for both UCHL3 (Pan *et al.*, 2019) and the NZF domain of TAB2 (Li *et al.*, 2021).

### Cell-type dependent interactions

Another issue that we addressed in **chapter 3** is the cell-type dependent and independent interactions that we observed when comparing embryonic stem cells (ESCs) and neuronal precursor cells (NPCs). In ESCs, we identified 156 cell-type-specific interactions and in NPCs 51 interactors specific for that cell-type. Subsequent absolute quantification of protein expression levels indicated that this wasn't due to high differences in protein expression levels, although some examples for which this is the case do exist (e.g. USP38, which binds $^{K29}Ub_2$ in ESCs, is 7-fold more abundant in ESCs than in NPCs). However, as can be seen in supplementary data available in the online paper (Table S1) and in supplementary figure S4C, some interactors become more specific for a certain ubiquitin linkage type in different cells or completely change specificity. Interesting examples in this respect are for instance Vps37A, RIOK3 and UBAC2. Vps37A, a component of the ESCRT-I complex and involved in vesicular trafficking, binds to K48-linked diubiquitin in all cell types tested, but additionally changes specificity from K27- to K6-linked diUb for HeLa and ESCs respectively. RIOK3, for which we identified the MIU (domains) as a potential M1 and K6 interactor in **chapter 2**, does not show a great amount of binding to diUbs in HeLa cells, but becomes a strong interactor for $^{K6}Ub_2$ in both ESCs and NPCs. Finally, UBAC2, which contains an UBA domain identified as a strong $^{K6}Ub_2$ binder (**chapter 2)**, shows no binding to $^{K6}Ub_2$ in HeLa cells, but in ESCs becomes equally specific for K6- and K48-linked diubiquitin (table 3). This virtuosity can for instance be explained by differences in adaptor proteins present in different cell types

and/or by additional post-translational modifications on the full-length ubiquitin-binding proteins themselves.

| | UBAC2 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **K6** | **K11** | **K27** | **K29** | **K33** | **K48** | **K63** | **Met1** | **Mono** | **Control** |
| **HeLa** | -0,16 | -0,19 | -0,04 | -0,66 | 0,52 | 1,10 | 0,40 | 0,65 | 0,32 | -1,93 |
| **ESC** | 1,46 | -0,01 | -1,01 | 0,36 | -0,16 | 1,45 | 0,46 | 0,42 | 0,43 | -3,40 |
| **NPC** | -0,04 | -0,69 | -1,09 | 0,11 | 0,67 | 1,95 | 0,80 | 0,82 | 0,53 | -3,07 |

***Table 3. Ubiquitin linkage-specific pulldown profile for UBAC2 in different cell types.*** *Displayed is the average log2 enrichment for each diubiquitin type possible and mono Ub. Abbreviations: ESC = embryonic stem cells, NPC = neuronal precursor cells*

## Ubiquitin linkage distribution in different cell different cell and tissue types

While the effects of adaptor proteins and post-translational modifications undoubtedly play a significant role, another important point to consider in this respect is the relative presence of differently linked ubiquitin chains in different cell and tissue types. For instance, the distribution of lysine Ub linkages varies greatly between the yeast *S. cerevisiae* and the commonly used HEK293 cells (Xu *et al.*, 2009; Dammer *et al.*, 2011). While in yeast different Ub lysine linkages are more diversely distributed, the distribution shifts more towards K48- and K63-linked ubiquitin chains in the rapidly dividing HEK293 cells (Fig. 1A). Additionally, K48-linked chains were found to be elevated in mouse liver tissues while K63-linked chains were relatively elevated in mouse cortex and lung (Dammer *et al.*, 2011). In bone marrow-derived macrophages, the population of polyubiquitin linkages mainly consists of K29- (8 %), K48- (63.2 %) and K63-linked (24.3 %) ubiquitin (Heunis *et al.*, 2020). Interestingly, the authors also report on Ub linkage distribution in different mouse tissues and find that K33-linkages are highly elevated in heart and muscle tissue (Fig. 1B), further outlining the importance of considering which tissue or cell-type is used.

## Stimulus dependent interactions

The effect of the cell-type used and the ubiquitin-linkage distribution within can be influenced further by different external stimuli and/or changes in cellular processes. For instance, stimulus-dependent poly-Ub modifications can be found on EGFR, which is rapidly modified with K63-linked chains upon stimulation with EGF (Tsuchiya *et al.*, 2018). Another example is RIP1, which is primarily ubiquitinated by the K63-linkage type upon stimulation with TNFα (Mevissen *et al.*, 2013). Stable isotope labeling with amino acids in cell culture (SILAC) demonstrated that K11 linkages are > 2 times elevated in rapidly dividing HEK293 cells compared to mouse lung, cortex, muscle, liver and kidney tissues (Dammer *et al.*, 2011): an observation that corresponds well with the crucial role for K11-linked chains in cell division (Matsumoto *et al.*, 2010). Also mitochondria become decorated with K6, K11, K48 and K63 ubiquitin linkages upon mitochondrial damage in HeLa cells (Ordureau *et al.*, 2014) and with K6, K11 and K63 linkages in neuronal SH-SY5Y cells (Cunningham *et al.*, 2015). As discussed in **chapter 5**, many proteins playing a role in the innate immune response become

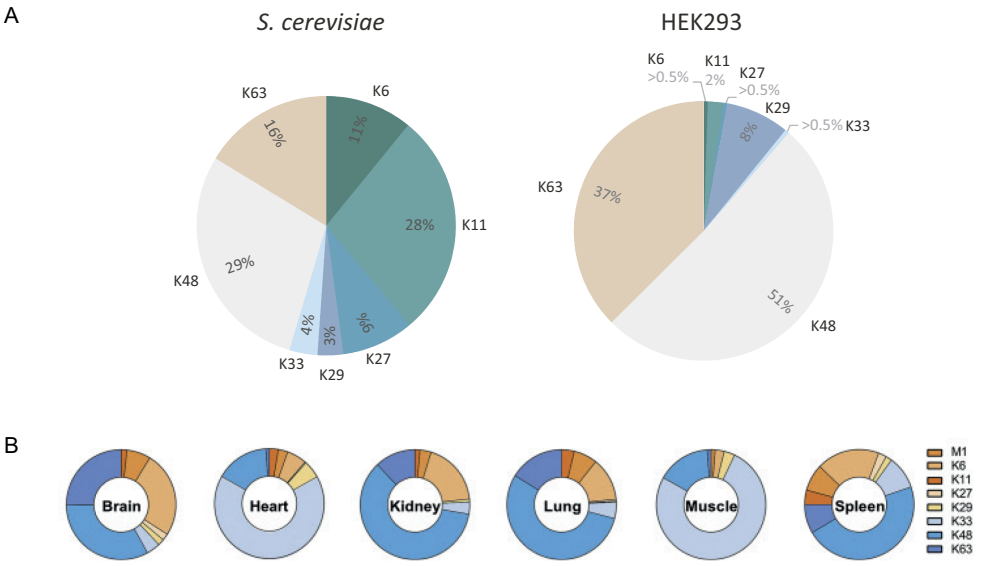


***Figure 1. Ubiquitin linkage distribution in different cell types and tissues.*** *A) Relative distribution of lysine-linked Ub in yeast and mammalian HEK293 cells. The amounts represented here were normalized against the total amount of lysine-linked ubiquitin. Adapted and modified from Xu et al., 2009 and Dammer et al., 2011 . B) Overview of the distribution of all eight Ub-linkage types in different mouse tissues (Heunis et al., 2020).*

K27-ubiquitinated upon viral infection. Considering the normally low abundance of K27-linked chains in most cell types and tissues (Fig. 1), it is likely that viral infections result in an overall cellular increase in K27-linked chains. This in turn becomes relevant considering the cumulative probability mechanism of UCHL3 inhibition when K27-linked ubiquitin chains are in abundance.

In **Fig. 6** of **chapter 3**, we investigated changes in K6-binding properties of proteins involved in the DNA damage response upon induction of double strand breaks by the DNA damage-inducing agent doxorubicin. We identified 10 interactors whose binding to K6-linked diubiquitin increases upon doxorubicin treatment, including the two known DNA-damage related proteins CDK7 and CDKN2A. In this context, UBXN1, which we investigated for its K6 binding properties in **chapter 2**, could also be an interesting protein to investigate. UBXN1 has been previously reported as an adaptor protein for the BRCA1-BARD1 complex and binds to auto-ubiquitinated K6-linked polyubiquitin chains on BRCA1 (Wu-Baer, Ludwig and Baer, 2010). In a recent publication we confirmed this specificity for K6-linked diubiquitin and showed that the canonical UBA domain alone is not sufficient for $^{K6}Ub_2$ binding (Shahul Hameed *et al.*, 2019). In **chapter 2** we further investigate the potential mechanism underlying this specificity by NMR. Interestingly, UBXN1 is also present in our linkage-specific pulldown efforts presented in **chapter 3**. Similar to UBAC2, we observe more binding to $^{K6}Ub_2$ in ESCs than in the other cell types tested (table 4). Since the BRCA1-BARD1 dimer is involved in repair of double-strand DNA breaks (Moynahan

and Jasin, 2010), one could expect the interaction with UBXN1 to be more prevalent in DNA damage induced cells. Wu-Baer *et al.* however already state that even though the ubiquitin binding properties of UBXN1 are required for BRCA1 inhibition, the interaction between the two proteins does not have an effect on homology-directed repair of double strand DNA breaks itself. This could also explain why we do pull down UBXN1 upon doxorubicin treatment from cell lysates, but only marginally enriched with respect to untreated cells (0.43-fold enrichment over non-treated cells (log2), Table 5). As expected, UBAC2 is not enriched in these pulldown experiments (chapter 3, online supplementary table S1), indicating that we mostly enrich proteins involved in the DNA damage response.

| | UBXN1 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **K6** | **K11** | **K27** | **K29** | **K33** | **K48** | **K63** | **Met1** | **Mono** | **Control** |
| **HeLa** | 0,05 | 0,15 | 0,02 | -0,37 | 0,50 | 2,25 | 0,16 | 0,53 | -0,31 | -2,99 |
| **ESC** | 0,82 | -0,15 | -0,82 | 0,26 | 0,02 | 2,45 | 0,21 | -0,42 | -0,26 | -2,11 |
| **NPC** | 0,17 | -0,26 | -0,70 | -0,05 | 0,00 | 2,39 | 0,25 | 0,22 | -0,14 | -1,88 |

| | UBXN1 | | | | |
|---|---|---|---|---|---|
| | **Control** | **Mono** | **Doxo_Mono** | **K6** | **Doxo_K6** |
| **HeLa** | -3,27 | 0,58 | 0,95 | 0,66 | 1,09 |

***Table 4 and 5. Pulldown data from chapter 3 for UBXN1.*** *Top panel: UBXN1 linkage-specific binding in different cell types. Displayed is the average enrichment over the row mean (log2) for each diubiquitin linkage type possible. Bottom panel: Average enrichment (log2) of UBXN1 for mono ubiquitin and K6-linked diubiquitin in doxorubicin treated HeLa cells versus wild-type HeLa cells.*

## The advantages and disadvantages of screening cell lysates versus ubiquitin binding domains

The major advantage of a cell lysate-based approach is the ability to measure linkage-specific interactions for proteins containing multiple ubiquitin binding domains. It also allows investigation of ubiquitin binding properties in different cellular contexts and with different (external) stimuli. On the other hand, screening individual binding domains may not always seem to be representative in terms of cellular context, but the possibility that the right cell-type and signaling pathway have not been examined is something to keep in mind. In addition, screening individual UBDs provides insight into sequential amino acid composition that could provide clues into underlying factors of ubiquitin linkage-selectivity. Finally, transient interactions with low affinity, as is often the nature for UBDs, are more easily picked up.

## FUTURE PERSPECTIVES FOR DISCOVERING UBIQUITIN LINKAGE-SPECIFIC BINDING

### Using UBDs as TUBEs to search for ubiquitylated substrates of a particular linkage specificity

Many ubiquitin binding domains have been transformed into tandem ubiquitin binding entities (TUBEs) to isolate (poly)ubiquitinated substrates from cellular lysates (Mattern *et al.*, 2019; Kliza and Husnjak, 2020). This also includes a few reported linkage-specific UBDs meant to detect or enrich for a certain ubiquitin linkage type, such as the K29/K33-specific NZF TRABID domain, the K63-specific NZF TAB2, UIM RAP80 and UIM VPS27 domains, and finally the M1-specific UBAN domains (Emmerich and Cohen, 2015). While TUBEs can be very useful tools, it is wise to keep in mind that UBDs often do display binding behavior towards other ubiquitin linkages to a certain degree (**chapter 2**). This can for instance become of relevance in cell types with low abundant distribution of certain linkage types, as discussed earlier on in this chapter. Importantly, not all reported linkage-specific UBDs were tested against all ubiquitin variants possible because of unavailability issues at the time. For instance, the NZF domain of TAB2 is widely known as K63-specific (Kulathu *et al.*, 2009; Sato *et al.*, 2009), but was later shown to bind with slight preference / equal affinity to K6-linkages as well (**chapter 3**; Li *et al.*, 2021). Cross-reactivity will likely continue to play a role in both TUBEs and in ubiquitin linkage-specific antibodies and is an important aspect to keep in mind while using them for experiments. Nevertheless, accompanied with proper controls, using linkage-specific UBDs as TUBEs or as cell-based sensors provides a powerful addition to the growing toolbox that can be used to unravel the ubiquitin landscape. In that respect, the K6-linkage specific UBDs that we identified in **chapter 2** could provide interesting opportunities as TUBEs, although their composition and/or the experimental design should be further optimized.

## Phosphorylated UBDs

Over the last years, reports have trickled in on the existence of phosphorylated ubiquitin binding domains. In 2011, Matsumoto *et al.* showed that the UBA domain of p62, also known as SQSTM, can be phosphorylated on S403 by casein kinase 2 (CK2) to increase its binding capacity to polyubiquitin chains (Matsumoto *et al.*, 2011). The event enhances recruitment of poly-ubiquitinated proteins into sequestosomes that serve as entry points for the autophagy pathway. In contrast to the wild-type UBA domain, phosphorylated UBA (pUBA) not only increases the preference for K63-linked ubiquitin chains but also for branched K48- and K63-linked polyubiquitin chains. A later study reported additional phosphorylation of the p62 UBA domain at S407 by ULK1 kinase, which in turn destabilizes the UBA dimer interface and thus increases its binding capacity to ubiquitin (Lim *et al.*, 2015). Interestingly, mutational analysis suggested that phosphorylation at S407 is required for S403 phosphorylation, which can also be catalyzed by ULK1. A subsequent study reported that the bi-phosphorylated UBA domain bound better to $^{K63}Ub_2$ than mono-phosphorylated UBA did (Tan *et al.*, 2017).

Another UBD that can be phosphorylated concerns the UBAN domain of optineurin (OPTN): like p62 also a receptor for the autophagy pathway (Richter *et al.*, 2016). TBK1 (Tank binding kinase 1) phosphorylates multiples sites in OPTN, but only S473 was found to enhance binding to cellular polyubiquitinated substrates. S473 phosphorylated UBAN (pUBAN) also bound significantly stronger to tetra-Ub chains than wild-type UBAN domain did. In addition, many diubiquitin variants (with the exception of $^{K11}Ub_2$ and $^{K48}Ub_2$) suddenly gain the capacity to bind the phosphorylated UBAN domain as well. Strikingly, pUBAN also bound

stronger to S65 phosphorylated mono ubiquitin, thereby implicating it in PINK1-dependent and PARKIN-independent mitophagy.

It will be interesting to see if more phosphorylated UBDs exist and if they can enhance or change ubiquitin (linkage-specific) binding. Insertion of phosphorylated residues during solid-phase peptide synthesis is relatively straightforward (Xu *et al.*, 2020) and complemented by fluorescence polarization screening could provide interesting and novel insights into this relatively uninvestigated area of research.

### Chain-length dependent interactions and mixed chain pulldowns

With regard to the identification of linkage-specific ubiquitin binding proteins from cell lysates there are still plenty of opportunities ahead. Ubiquitin chain length can definitely influence and/or enhance linkage-specific UBD or UBP binding, as discussed in **chapter 2** and **3**. Recently, Lutz *et al.* showed that there indeed are many proteins that bind to polyubiquitin chains longer than 6 entities specifically (Lutz *et al.*, 2020). However, they also find a class of ubiquitin-binding proteins that interact only with shorter K29- and K33-linked chains (di- and tetra-Ub) and suggest that the shorter ubiquitin variants represent an unique class of conformations that cannot be adopted by longer polyUb chains. Although it is generally assumed that short ubiquitin chains such as dimers do not exist in the cell, this finding makes one wonder if perhaps there is a role for them after all.

The study by Lutz *et al.* only focused on K27-, K29- and K33-linkages and it will be interesting to see what kind of interactors can be identified for other homotypical ubiquitin linkage types of a higher order. This also applies for heterotypical chains, branched ubiquitin chains, post-translationally modified ubiquitin chains and Ub/UbL mixed chains and it is clear that there is still much to learn in this area.

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