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Systematic Yeast Two-Hybrid Analysis of Human E2 Ubiquitin-Conjugating Enzyme and Deubiquitin (DUB) Protein Interactions

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ABSTRACT

Post-translational modification of proteins via ubiquitination is mediated by three enzyme families; E1 activating enzymes, E2 conjugating enzymes and E3 ligases, all of which work in a hierarchical manner to facilitate different forms of protein ubiquitin ranging from mono-ubiquitination to the formation of different forms of ubiquitin chains. Reversibly, deubiquitinating enzymes (DUBs) act to remove ubiquitin from modified substrates. Apart from the classic interactions within the E1-E2-E3 enzymatic cascade, an unusual non-hierarchical interaction has been observed between some E2 enzymes and a DUB called Otubain-1 (OTUB1). This observation raises interesting questions concerning the order and specificity within the human ubiquitin system. In this study, systematic yeast two-hybrid (Y2H) binary screen is performed between 39 E2 and 60 DUB proteins to analyze the extent of human E2-DUB interactions. As a result, putative partnerships between OTUB1 and UBE2D1, UBE2D2, UBE2D3, UBE2D4, UBE2E1, UBE2E2, UBE2E3 and UBE2N are identified and these data correlate well with data from other independent study by high-throughput Y2H library screen and mass spectrometry. In essence, this study confirmed that E2-DUB interactions within the human ubiquitin system are indeed uncommon and only unique to OTUB1 protein.

Key words: Ubiquitination, non-hierarchical, human E2, DUB, yeast two-hybrid

INTRODUCTION

Protein ubiquitination is a complex enzymatic post-translational modification process that is carried out by a combinatorial action of E1, E2 and E3 proteins. It is a reversible process that regulates a myriad portfolio of cellular processes ranging from mitosis (Morgan, 1999), apoptosis (Fang *et al*., 2000), gene regulation (Fang *et al*., 2004; Eskeland *et al*., 2010), response to infection (Ozato *et al*., 2008; Carthagena *et al*., 2009) and DNA damage (Nakada *et al*., 2010). In this process, a small 8.5 kDa regulatory protein aptly named ubiquitin is attached to protein substrates thereby labeled them either for destruction or direct them to other locations in cell to perform many other cellular processes (Ciechanover, 1998; Weissman, 2001). The multi-stage ubiquitination cascade is initiated by the E1 activating protein which generates a high-energy thioester intermediates E1~ubiquitin molecule in an ATP-dependent manner, thereby conditioning the ubiquitin to be passed onto the catalytic cysteine residue of an E2 conjugating enzyme. The two main families of E3 ligase protein; HECT and RING finger proteins catalyze the transfer of ubiquitin to specific target substrates (Metzger *et al*., 2012). The HECT ligases recognize and bind ubiquitin to form E3~ubiquitin intermediate before transferring it onto the target substrate protein

(Huang *et al*., 1999). Meanwhile, RINGs act mainly as a molecular scaffold that brings the E2 and substrate into close proximity hence ubiquitin could be transferred directly from the E2 to the substrate. The ubiquitination process can either forms mono-ubiquitinated substrate or is repeated until a short polyubiquitin chain is form. Ubiquitin pathway is negatively regulated by a large group of deubiquitinases (DUB) which cleave off the ubiquitin-protein bonds and by that means reversing the ubiquitination message (Komander *et al*., 2009).

The multifarious activity of ubiquitin pathway is determined by its seven lysine residues, each of which can potentially mediate attachment to other ubiquitin molecules, allowing the formation of a range of structurally distinct polyubiquitin chains (Komander, 2009). The lysine selection is performed by an E2 enzyme which specifies ubiquitin chain architecture for example Lys48-linked chains label proteins for proteasomal degradation (Chau *et al*., 1989), whereas Lys63 ubiquitin tag promotes protein trafficking, kinase activation and proteolytic degradation of misfolded proteins, to name but a few (Olzmann *et al*., 2007; Tan *et al*., 2008; Wooten *et al*., 2008). Hence, the variety of ubiquitin chain topology bears diverse implication in biological processes (Komander *et al*., 2009).

Detailed analysis of protein interaction preferences within the human ubiquitination process is crucial in order to provide a better understanding of the order and specificity within this system. High-throughput yeast two-hybrid (HTP-Y2H) library screen has identified a surprising interaction involving specific E2 proteins (UBE2D1, UBE2D2, UBE2D3 and UBE2E1) and a DUB called Otubain-1 (OTUB1) (Markson *et al*., 2009-Suppl File 2). Additionally, other independent mass spectrometry analysis revealed that OTUB1 forms complex with UBE2D2 and UBE2N (Sowa *et al*., 2009). These data raised an interesting question as to why OTUB1 should associates with specific E2 proteins. Classically, DUB proteins are well known for cleaving or trimming ubiquitin chains from the substrates and had been found to interact with a number of E3 proteins. On the other hand, association of DUB and E2 enzymes is very rarely observed. Structural investigation of E2-OTUB1 interactions revealed that OTUB1 inhibits ubiquitin binding to E2 enzymes in non-catalytic manner. The E2 is recognized by OTUB1 through contacts with both donor ubiquitin and the E2 enzyme in which, OTUB1 binds preferentially to this ubiquitin-charged E2. By mimicking the Lys48-linked ubiquitin recognition, another free ubiquitin interacts with the N-terminal ubiquitin-binding site on OTUB1 to promote binding with the ubiquitin-charged E2 protein (Juang *et al*., 2012). Thus, apart from its canonical isopeptidase activity, OTUB1 also accomplishes its function as deubiquitin enzyme by blocking ubiquitin chain synthesis through binding with E2 enzymes.

Yeast two-hybrid (Y2H) is a powerful tool that has been extensively used to study protein-protein interactions as it can be performed in a high-throughput format. Great advantage of this technique is that it could detect even transient or weak binary protein interactions. In this system, a protein of interest (the bait) is fused to the DNA Binding Domain (BD) of a transcription factor (such as GAL4) while the bait's potential interacting partner (the prey protein) is fused to the transcription factor's Activation Domain (AD). These fusions are carried out by DNA cloning methods, allowing expression of the subsequent bait and prey fusion proteins in the nucleus of the yeast host. The yeast strain used in this system carries a set of reporter constructs that are under the control of an upstream sequence containing the binding sites for the BD. If the bait-BD and prey-AD fusions interact, then a functional transcription factor is reconstituted and expression of the reporter gene is activated (Fields and Song, 1989).

In this study, Y2H matrix method is employed with the aim to establish if OTUB1 was unique among DUB enzymes in being able to bind E2 proteins and to verify that OTUB1 binds E2 proteins

via direct binary interactions in order to support the existing Y2H library screen and mass spectrometry study (Markson *et al.*, 2009; Sowa *et al.*, 2009). Finally, the ultimate aim of this project is to identify more E2-DUB partnerships and to confirm on which are already discovered.

MATERIALS AND METHODS

Construction of Y2H clones: pGBAD-B and pACTBD-B vectors were used to construct sets of E2 bait and prey clones. For DUB proteins, baits were inserted in pGBDU-GW and preys were expressed in the pACTBE-B vector. All bait and prey clones were generated using established high throughput gap repair and yeast transformation reactions described elsewhere (Semple *et al.*, 2005). In general, gene specific inserts were amplified by proofreading PCR reactions from available pDONR223 entry clones. Following gap repair and yeast transformation, clone identity was verified by PCR and each clone was assessed for autoactivation. Haploid yeasts which grows on media lacking either histidine (-His) or adenine (-Ade) must be autoactivating the HIS3 or ADE2 reporters independently hence were eliminated from further studies.

In this experiment, PJ69-4A (MATa trp1-901 leu2-3, 112 ura3-52, his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) was used as the bait strain and PJ69-4α, a suitable mating partner with identical genotype to PJ69-4A was used as the prey strain (James *et al.*, 1996). The complete set of human E2 and DUB clones used in this experiment are listed in appendices 1 and 2.

Yeast two-hybrid matrix mating: E2 bait clones (in pGBAD-B) were mated with an array of human DUB prey clones (in pACTBE-B) on YPAD media and incubated for 24 h at 30°C. All Y2H assays performed in this study employed a mating strategy to generate diploid yeast containing both bait and prey constructs. This procedure has been shown to be more efficient than co-transfection protocols which tend to be affected by variability in transfection efficiency (Garcia-Cuellar *et al.*, 2009).

The colonies were replicated on a synthetic dropout (SD)-Trp/Leu media using a sterile velvet cloth and incubated for 48 h at 30°C to select diploid colonies. The final transfer was performed onto a triple dropout media (SD-Trp/Leu/His containing 2.5 mM 3-AT or SD-Trp/Leu/Ade) to screen for activation of reporter genes (HIS3 and ADE2). The colony growth was scored for up to 11 days. The same protocol was repeated for mating a set of DUB bait clones (in pGBDU-GW) with collection of human E2 prey clones (in pACTBD-B). In contrast with E2 bait-DUB prey assay, the diploid screening for DUB bait-E2 prey assay was done on SD-Ura/Leu since pGBDU-GW vector carries URA3 marker. Apparently, the final screens were also need to be performed on SD-Ura/Leu/His (+2.5 mM 3-AT) and SD-Ura/Leu/Ade plates. The activation of the reporter genes ADE2 and HIS3 produces a scorable phenotype on either -His or -Ade plates or both, in which the number of yeast colonies were counted to define the strength of particular interactions.

Pooling-deconvolution strategy: Y2H experiment between E2-DUB set was done using a pooling strategy to obtain a comprehensive interaction profile since this strategy could dramatically decrease effort required to perform large-scale Y2H screens. It also allows simultaneous screening of multiple prey constructs with each bait clone, thus enabling a greater number of possible interactions to be screened in fewer experiments to provide greater screen coverage. Yeast colonies were arrayed in 96 well plate format (8×12), therefore pooled mating assays were performed by picking yeast colonies from each vertical row and combining them together in one tube. Hence, for

the full 96 well plate, 12 pools were generated representing each column. Consequently, these pools were mated against specific bait. Pools with positive interaction profiles were then deconvoluted and mated with the same bait clone that gave positive results in initial pooled screens.

RESULTS AND DISCUSSION

Analysis of the Y2H data: In this experiment, 30 DUB preys pooled into 12 separate groups were screened in duplicate against 39 E2 baits. Using this approach, only 468 mating were needed to test 1170 potential binary interactions. From these screens, 24 positive interactions were detected and deconvoluted resulting in 15 E2-DUB interactions, most of which involved OTUB1 which interacted with all members of the E2 from the D and E subfamilies (with the exception of UBE2E2) and UBE2N, as seen in Fig. 1a-b. Other positive interactions were observed between UBE2U, TSG101 and UBE2DNL which share common DUB interactions; COPS6 and EIF3F. EIF3F also interacted with AKTIP.

Meanwhile, 39 E2 preys were pooled into 12 groups and mated against 60 individual DUB baits. In this assay, 2340 binary interactions were tested in 720 pooled mating. In this orientation, less positive hits were observed with only 12 positive interactions being identified. The deconvolution mating of selected E2 pool preys against DUB baits resulted in 12 binary E2-DUB interactions that activated HIS3, ADE2 or both reporters. OTUB1 was again the major interactor for the E2s, with obvious positive interactions with UBE2D2, UBE2E1, UBE2E2, UBE2E3 and UBE2W. Besides, other partnerships were observed between TNFAIP3-UBE2I, TNFAIP3-UBE2U and USP2a-UBE2U (Fig. 1c-d).

Usually, an interaction is considered to be a true positive only if it activated all three GAL4 reporter genes: HIS3, ADE2 and lacZ but based on experience, the lacZ reporter in the PJ69-4 strains appears to be less reliable than the two growth reporters. Therefore, this study independently monitors growth on -His or -Ade conditions for true interaction. Although, growth on both independent reporters is considered ideal, previous undertaken experiments show that reproducible positive interactions observed on -His selection alone were sufficient to allow the reliable prediction of interactions provided they are then confirmed by subsequent *in vitro* assays or mutagenesis studies (Markson *et al.*, 2009). In some cases, irreproducible positive interactions could also be eliminated because only reproducible interactions detected in at least two independent assays were counted as true positive interactions.

From the analysis, it appears that E2-DUB pairs are very uncommon in cells because from a total of 3510 binary interaction tested, only 27 positive hits were identified. However, this low score of positive results may also due to the restricted number of DUBs tested in this Y2H screen. As our final DUB bait and prey clone sets are not totally comprehensive clone sets (as listed in appendix 2), it is possible that several potential interactions may have been missed. From the 27 hits, 12 are involving OTUB1 and more importantly, only OTUB1 gave a strong, confident and reproducible results in both bait and prey direction while other interactions were rather weak and only detected in one of the bait prey orientation. Therefore, it is worth noting that most interactions apart from OTUB1 could not be confidently ascertained as real interactions even though they produce positive colony growth.

Y2H screen identified strong, binary interactions between OTUB1 and E2 proteins from the D and E subfamilies: OTUB1 shows a clear binding preference for E2 proteins from the D and E subfamilies and UBE2N. Interestingly, the E2-conjugating D subfamily is widely known as the most promiscuous E2 protein (Brzovic and Klevit, 2006) and contributed to major E2-E3 RING partnership in HTP-Y2H library assay (Markson *et al.*, 2009). This may be consistent with their

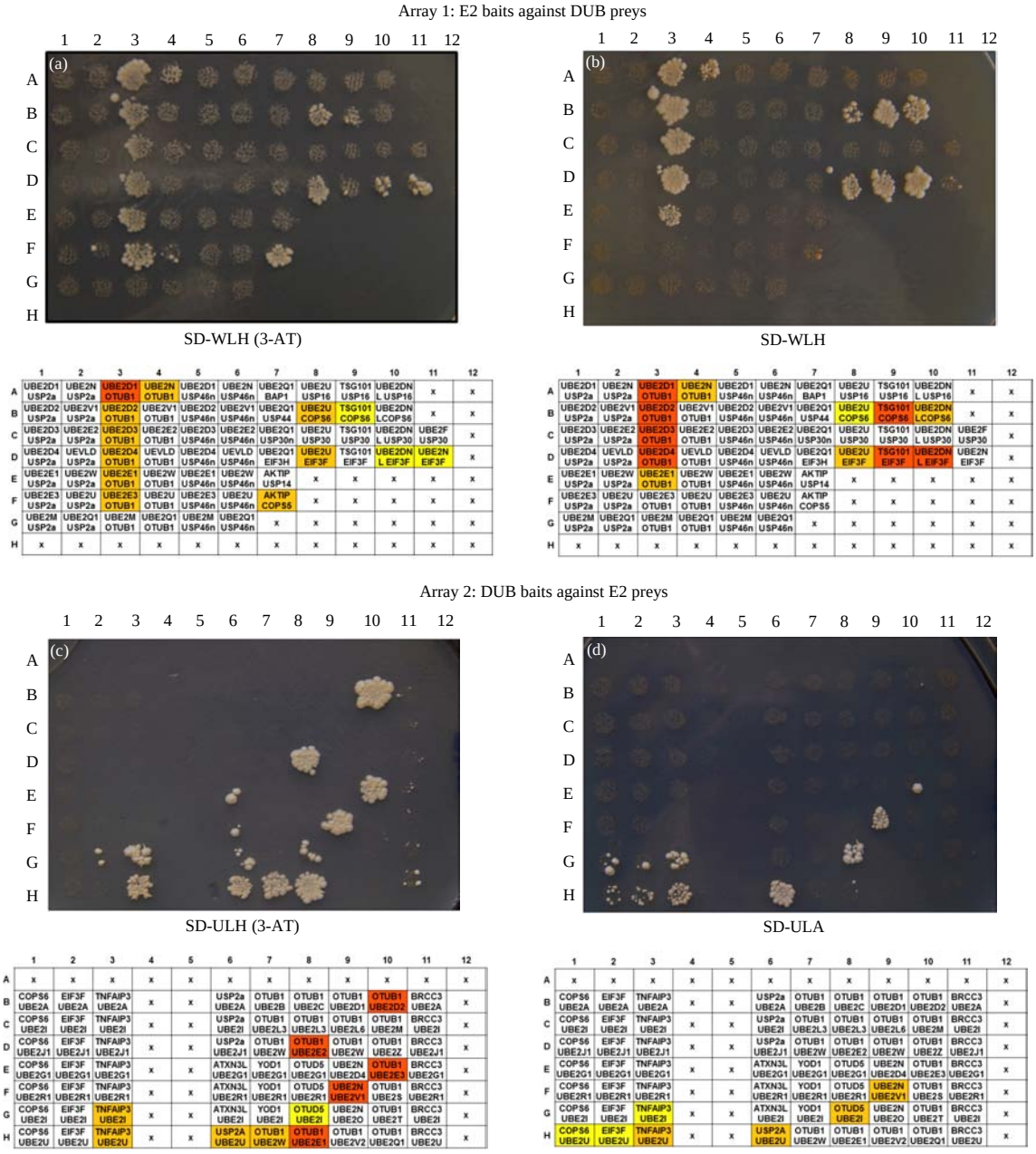

Fig. 1(a-d): Screens of E2-DUB pooled preys deconvoluted in singe mating. Y2H screens were performed on -His and -Ade triple dropout media. Colony growth is scored YELLOW for weak interaction (5-19 colonies), ORANGE for medium interaction (20-200 colonies) and RED for strong interaction (≥200 colonies/full plaque)

role as housekeeping E2s within the ubiquitin system. As seen in Fig. 2, members in D subfamily are highly similar in their primary sequence thus more often than not, exhibit common patterns of interaction. The D subfamily also shares a high level of primary sequence similarity with E subfamily members; however, the latter contain an approximate 60 amino acids N-terminal

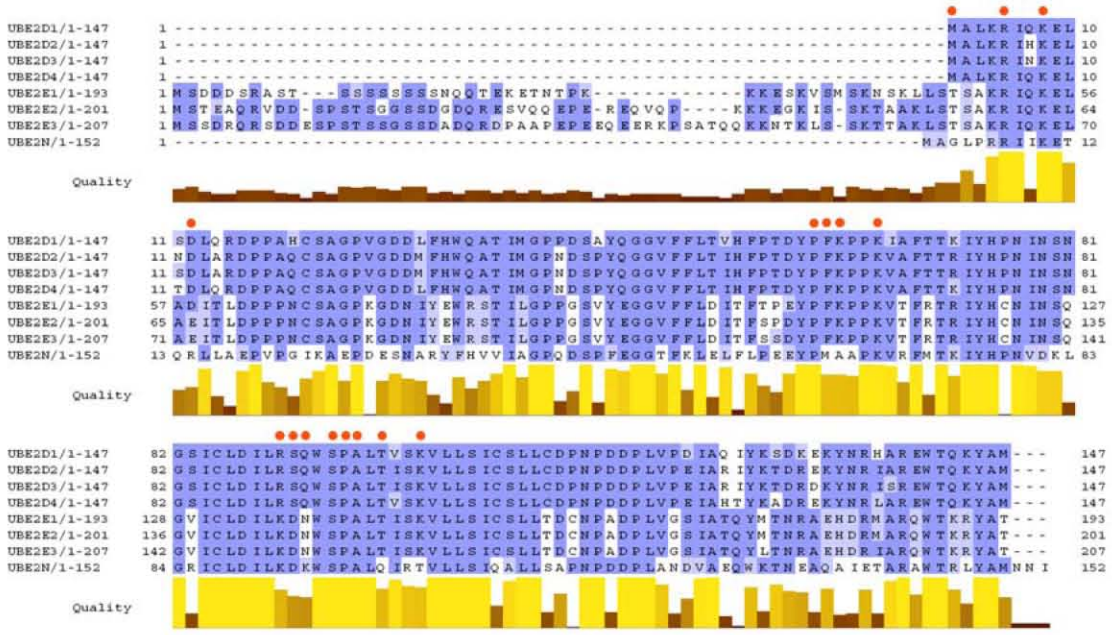

Fig. 2: Sequence alignment of E2 D and E subfamilies and UBE2N. The sequences are colored according to Blosum62 as well as the alignment quality. Red dots indicate the UBE2D2 residues interacting with OTUB1 (Juang *et al.*, 2012)

extension not found in D subfamily E2 proteins. These extensions are thought to be influential in determining specificity as E subfamily members are less promiscuous than UBE2D proteins. Due to this high resemblance, it is expected that both E2 subfamilies share a similar interface in binding with OTUB1.

In forming ubiquitin chains, UBE2Ds, UBE2Es and UBE2N show a diversity of preferences. D and E subfamilies are more promiscuous and can catalyze the formation of multiple linkage chain types (Kirkpatrick *et al.*, 2006; Kim *et al.*, 2007; Jin *et al.*, 2008). In addition, promiscuous UBE2Ds preferentially promote the formation of Lys11-, Lys48- and Lys63-linked chains in vitro with three different E3s and show evidence of mixed and branched chains (Kim *et al.*, 2007). Meanwhile, a dimeric complex composed of UBE2N and UBE2V1 was determined to only form Lys63 linkage specificity (VanDemark *et al.*, 2001; Eddins *et al.*, 2006). The fact that UBE2Ds, UBE2Es and UBE2N forming different chain topologies indicate that OTUB1-mediated inhibition of the E2 may involves in various ubiquitination events.

Insight into other E2 interactors apart from OTUB1: An interesting partnership is observed between COPS6 and TSG101, as both of these proteins are involved in interactions with p53 (Li *et al.*, 2001; Bech-Otschir *et al.*, 2001). TSG101 is an E2 belonging to UEV (ubiquitin E2 variant) domain members which shows significant sequence similarity to E2 enzymes. However, they are unable to catalyze ubiquitin transfer as they lack the active site cysteine that forms the transient thioester bond with the C-terminus of ubiquitin (Koonin and Abagyan, 1997; Ponting *et al.*, 1997). In relationship with p53, TSG101 participates with E3 ligase MDM2 in an autoregulatory loop that modulates the cellular levels of both proteins and of p53. Meanwhile, COPS6 (also known as CSN6) is one of the eight subunits that make up the COP9-signalosome, a

highly conserved protein complex that function as an important regulator in multiple signaling pathways (Wei *et al.*, 2008). Recently, another DUB component of the COP9 signalosome, COPS5 (or CSN5), has been shown to regulate p53 function (Zhang *et al.*, 2008) and p53 has also been shown to bind the native COP9 signalosome with high affinity through COPS5 (Bech-Otschir *et al.*, 2001). The observed interaction between COPS6 and TSG101 could therefore, represent a mechanism by which p53 activity or stability could be regulated. In this experiment, COPS6 also interacted with UBE2DNL, a pseudogene with UBE2D N-terminal like region but very few interactions or literature reports have been recorded for UBE2DNL hence the characteristic of this interaction could not be predicted.

UBE2I may have weak interaction with TNFAIP3 (also known as A20), functionally known as an inhibitor of cell death and chronic inflammation that downregulates NF-κB activation via the Tumor Necrosis Factor Receptor (TNFR)-associated pathway (Wertz *et al.*, 2004). TNFAIP3 is a very interesting protein because it is the only known DUB that also has E3 ligase activity mediated by one of its C-terminal zinc-finger domains that can promote the conjugation of Lys48-linked ubiquitin chains and proteasomal degradation (Wertz *et al.*, 2004). In order to disrupt interactions between E2:E3 enzymes in TNFR and the TLR4/IL-1R pathways, TNFAIP3 together with the regulatory molecule TAX1BP1 has been shown to interact with UBE2N and UBE2D3, the E2 involved in this pathway, thus triggering their ubiquitination and proteasome-dependent degradation (Shembade *et al.*, 2010). Interestingly, TNFAIP3 shares similarity with OTUB1 as both have OTU domains and both are known to be immunoregulatory DUBs (Sun, 2008). In this experiment, TNFAIP3, as well as USP2a, also interacted with UBE2U. However, no interaction data for UBE2U conjugation activity have been corresponded in the literature at present which may be related to its restricted expression pattern in the urogenital tract (Van Wijk *et al.*, 2009).

Unfortunately, apart from OTUB1, other DUB interactions observed in this study could not be treated as true interactions as they might be false positives and do not corresponded to known interactions found in human databases. Nevertheless, it offers novel candidates to be investigated in future interaction studies.

OTUB1 physiological functions: OTUB1, a cysteine protease initially found in ovarian tumors is a DUB that contains a conserved OTU (ovarian tumor) domain which is conserved in all OTUB proteins (Edelmann *et al.*, 2009). Its N-terminus contains a ubiquitin-binding domain which is thought to interact with ubiquitin to increase its binding affinity to E2 enzymes. OTUB1 was initially proposed to provide an editing function for polyubiquitin chain growth by cleaving tetraubiquitin substrate *in vitro* (Balakirev *et al.*, 2003). Surprisingly, it was later revealed that OTUB1 promotes rather than inhibits the K48-linked self-ubiquitylated and proteolysis of E3 RING ligase RNF128 (GRAIL) (Soares *et al.*, 2004). In the proteomic study by Edelmann *et al.* (2009), two more OTUB1 interactors were identified: FUS/TLS and Rack1, both of which are involved in RNA splicing. However, the significance of these interactions and whether FUS/TLS and Rack1 are deubiquitinated by OTUB1 remains unknown.

With a growing body of evidence that DUBs have non-canonical activity (Hanna *et al.*, 2006), OTUB1 proves this principle by its ability to regulate protein ubiquitination reactions. This is achieved by suppressing RNF168-dependent poly-ubiquitination by a mechanism that is independent of its catalytic activity, by simply binding to and blocking ubiquitin transfer and E3-RING docking to UBE2N, in other words they prevent ubiquitin attachment, rather than detaching bound ubiquitin, thereby inhibiting DNA repair (Nakada *et al.*, 2010; Sun *et al.*, 2012) and presumably many other cellular processes as well. The fact that OTUB1 inhibits DNA repair could have therapeutic relevance. Nakada *et al.* (2010) found that reducing the level of OTUB1

expression restores the process of homologous recombination in cells in which ATM (Ataxia telangiectasia mutated) kinase is inhibited. Thus, OTUB1 depletion can, in principle, mitigate DNA-repair effects. This observation makes the interaction between OTUB1 and UBE2N an attractive target for therapeutic intervention, with particular relevance for disorders affecting DNA repair and possibly for use in combination with radiation therapy.

OTUB1 inhibits Ub~UBE2N as well as Ub~UBE2D2 to attenuate DNA repair and induce apoptosis through p53 stabilization respectively (Wiener *et al*., 2012; Sun *et al*., 2012). As UBE2N and UBE2D2 are known to interact with a broad array of binding partners (Sowa *et al*., 2009), it is anticipated that OTUB1 inhibition will result in modulation of many cellular responses.

The UBE2D2 (and UBE2N) interaction with OTUB1 could be manipulated in establishing therapeutic drug targets to prevent OTUB1 binding and inhibiting the ubiquitination cascade. Being able to block OTUB1 would allow downstream cellular signaling pathways to occur. The involvement of UBE2D2 in the DNA damage response could be the target to allow the repair of DSBs and prevent chromosomal rearrangements that may lead to tumourigenesis and cancer. Although the downregulation of UBE2D2 by suppressing OTUB1 may seem promising, the analysis of OTUB1 network shows that it interacts with a wider range of proteins. Therefore, it should be considered whether the inhibition of OTUB1 would disrupt other physiologically important processes. Figure 3 shows the known OTUB1 interaction network that contains a broad range of

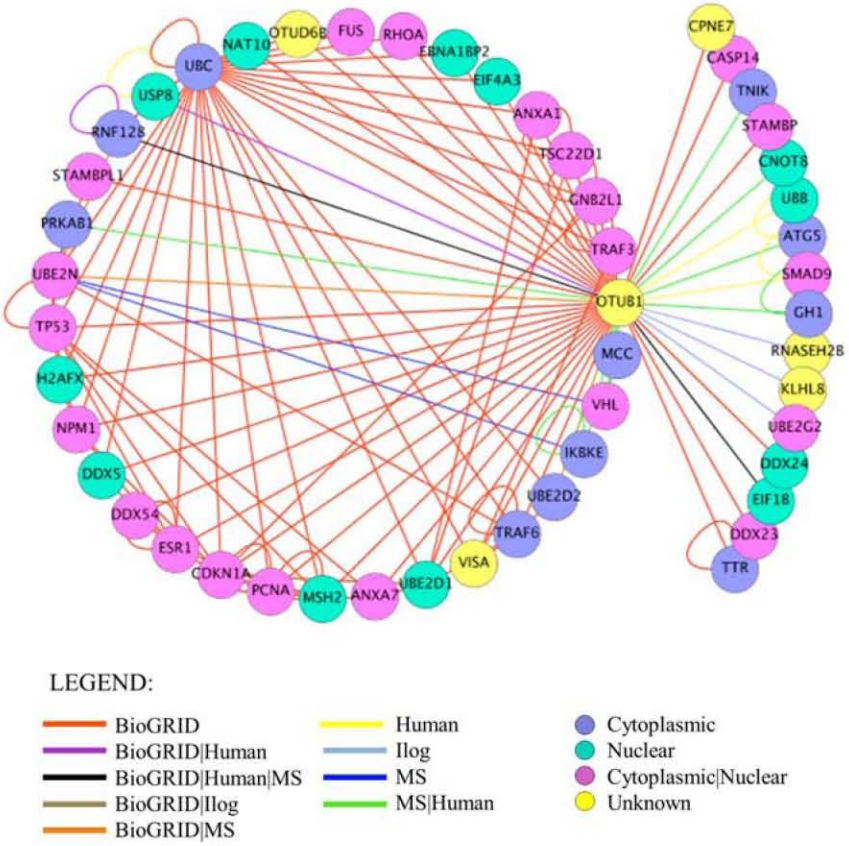

Fig. 3: OTUB1 interaction network. Edge color (line) indicates the data source or type of experiment performed in order to get interaction data. Nodes color (dot) indicates the localisation of each protein

binding partners, suggesting it has other function other than DNA damage response. Interestingly, several binding partners are known to have roles in the cell cycle which may represent an interesting area of future research.

CONCLUSION

Result obtained in this study confirmed that non-hierarchical interaction between E2 and DUB proteins within the human ubiquitin system is very uncommon. From the systematic Y2H binary analysis, it is proposed that OTUB1 was the only DUB that exclusively interacts with UBE2D1, UBE2D2, UBE2D3, UBE2D4, UBE2E1, UBE2E2, UBE2E3 and UBE2N. Although some weaker interactions involving other DUBs such as COPS6 and TNFAIP3 were also observed, they have a high possibility of being false-positive. Thus, it is strongly recommended that further biophysical or mutagenesis analysis to be performed to verify all the interactions observed in Y2H. Generation of the missing constructs of DUB bait and prey clones is also suggested in order to enable the screening of a greater number of possible interactions.

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APPENDICES

Appendix 1: E2-ubiquitin conjugating enzymes (E2s)

	Gene ID	Gene name	Alternate name	Bait (◆)	Prey (■)
1	7319	UBE2A	UBC2; HHR6A; RAD6A	◆	■
2	7320	UBE2B	HR6B; UBC2; HHR6B; RAD6B; E2-17kDa	◆	■
3	11065	UBE2C	UBCH10; dJ447F3.2	◆	■
4	7321	UBE2D1	SFT; UBCH5; UBC4/5; UBCH5A; E2(17)KB1	◆	■
5	7322	UBE2D2	UBC4; PUBC1; UBC4/5; UBCH5B; E2(17)KB2	◆	■
6	7323	UBE2D3	UBC4/5; UBCH5C; MGC5416; MGC43926; E2(17)KB3	◆	■
7	51619	UBE2D4	HBUCE1; FLJ32004	◆	■
8	7324	UBE2E1	UBCH6	◆	■
9	7325	UBE2E2	UBCH8; FLJ25157	◆	■
10	10477	UBE2E3	UBCH9; UbcM2	◆	■
11	140739	UBE2F	NCE2; MGC18120	◆	■
12	7326	UBE2G1	UBC7; E217K; UBE2G	◆	■
13	7327	UBE2G2	UBC7	◆	■
14	7328	UBE2H	UBC8; UBCH; UBCH2; E2-20K	◆	■
15	7329	UBE2I	P18; UBC9; C358B7.1	◆	■
16	51465	UBE2J1	UBC6; Ubc6p; CGI-76; NCUBE1; HSPC153; HSPC205; NCUBE-1; HSU93243; MGC12555	◆	■
17	118424	UBE2J2	NCUBE2; NCUBE-2; PRO2121BAIT	◆	
18	3093	UBE2K	LIG; HIP2; HYPG; UBC1; E2-25K; DKFZp564C1216; DKFZp686J24237	◆	■

Appendix 1: Continue

	Gene ID	Gene name	Alternate name	Bait (◆)	Prey (■)
19	7332	UBE2L3	E2-F1; L-UBC; UBCH7; UbcM4	◆	■
20	9246	UBE2L6	RIG-B; UBCH8; MGC40331	◆	■
21	9040	UBE2M	UBC12; hUbc12; UBC-RS2	◆	■
22	7334	UBE2N	UBC13; MGC8489; UbcH-ben; MGC131857	◆	■
23	63893	UBE2O	E2-230K; FLJ12878; KIAA1734	◆	■
24	55585	UBE2Q1	GTAP; UBE2Q; NICE-5; PRO3094	◆	■
25	92912	UBE2Q2	DKFZp762C143	◆	
26	997	UBE2R1	UBC3; UBCH3; CDC34; E2-CDC34	◆	■
27	54926	UBE2R2	UBC3B; CDC34B; FLJ20419; MGC10481	◆	■
28	27338	UBE2S	EPF5; E2EPF; E2-EPF	◆	■
29	29089	UBE2T	PIG50; HSPC150	◆	■
30	148581	UBE2U	MGC35130; RP4-636O23.1	◆	■
31	7335	UBE2V1	CIR1; UEV1; CROC1; UBE2V; UEV-1; UEV1A; CROC-1	◆	■
32	7336	UBE2V2	MMS2; UEV2; EDPF1; UEV-2; DDVIT1; EDAF-1; EDPF-1; DDVit-1	◆	■
33	55284	UBE2W	hUBC-16; FLJ11011	◆	■
34	65264	UBE2Z	USE1; HOYS7; FLJ13855	◆	■
35	100131816	UBE2DNL	MGC42638	◆	
36	55293	UEVLD	ATTP; UEV3; FLJ11068	◆	■
37	64400	AKTIP	FT1; FTS	◆	■
38	57448	BIRC6	BRUCE; APOLLON; FLJ13726; FLJ13786; KIAA1289	◆	■
39	7251	TSG101	TSG10; VPS23	◆	

The names and ID of the 39 human E2 and ubiquitin E2 variant (UEV) proteins involved in this experiment. Some of the constrnct are only available in bait as can be seen by legend: ◆ = bait; ■ = prey

Appendix 2: Deubiquitinating enzymes (DUBs) Y2H clone collection

	Gene ID	Gene name	Alternate name	Bait (◆)	Prey (■)
1	7345	UCHL1	PARK5; PGP95; PGP9.5; Uch-L1; PGP 9.5	◆	■
2	8314	BAP1	UCHL2; hucep-6; FLJ35406; FLJ37180; HUCEP-13; KIAA0272; DKFZp686N04275	◆	■
3	7347	UCHL3	UCH-L3	◆	■
4	51377	UCHL5	UCH37; CGI-70; INO80R; UCH-L5	◆	■
5	7398	USP1	UBP	◆	■
6	9099	USP2a	USP9; UBP41	◆	■
7	7375	USP4	UNP; Unph; MGC149848; MGC149849	◆	
8	8078	USP5	ISOT	◆	■
9	9098	USP6	HRP1; TRE2; TRE17; Tre-2; USP6-short	◆	
10	7874	USP7	TEF1; HAUSP	◆	
11	9101	USP8	UBPY; HumORF8; FLJ34456; KIAA0055; MGC129718	◆	
12	8237	USP11	UHX1	◆	
13	219333	USP12	UBH1; USP12L1	◆	■
14	8975	USP13	ISOT3; IsoT-3	◆	
15	9097	USP14	TGT	◆	■
16	9958	USP15	UNPH4; KIAA0529; MGC74854; MGC131982; MGC149838	◆	
17	10600	USP16	MSTP039, UBP-M	◆	■
18	10869	USP19	ZMYND9	◆	
19	10868	USP20	LSFR3A, VDU2	◆	■

Appendix 2: Continue

	Gene ID	Gene name	Alternate name	Bait (◆)	Prey (■)
20	27005	USP21	RP11-297K8.3, USP16, USP23	◆	■
21	29761	USP25	USP21	◆	■
22	83844	USP26	MGC120066; MGC120067; MGC120068	◆	
23	57646	USP28	KIAA1515	◆	
24	84749	USP30	FLJ40511, MGC10702	◆	■
25	84669	USP32	USP10; NY-REN-60	◆	
26	23032	USP33	VDU1; KIAA1097; MGC16868	◆	■
27	57602	USP36	DUB1	◆	
28	84640	USP38	FLJ35970; HP43.8KD; KIAA1891	◆	■
29	10713	USP39	SAD1; CGI-21; HSPC332; SNRNP65; MGC75069	◆	■
30	373856	USP41		◆	
31	84101	USP44	FLJ14528; DKFZp434D0127	◆	■
32	85015	USP45	MGC14793	◆	
33	64854	USP46	FLJ11850; FLJ12552; FLJ14283; FLJ39393	◆	■
34	84196	USP48	USP31; RAP1GA1; MGC14879; MGC132556; DKFZp762M1713	◆	
35	25862	USP49	MGC20741	◆	
36	9924	USP52	PAN2	◆	■
37	159195	USP54	C10orf29; FLJ37318; bA137L10.3; bA137L10.4	◆	
38	55611	OTUB1	OTB1; OTU1; HSPC263; MGC4584; FLJ20113; FLJ40710; MGC111158	◆	■
39	78990	OTUB2	OTB2; OTU2; MGC3102; FLJ21916; C14orf137	◆	■
40	55432	YOD1	DUBA8; OTUD2; PRO0907; DKFZp451J1719; RP11-164O23.1	◆	
41	54726	OTUD4	HIN1; DUBA6; HSHIN1; KIAA1046; DKFZp434I0721	◆	
42	55593	OTUD5	DUBA; MGC104871; DKFZp761A052	◆	
43	139562	OTUD6A	DUBA2; HSHIN6; FLJ25831	◆	
44	51633	OTUD6B	DUBA5; CGI-77	◆	
45	56957	OTUD7B	ZA20D1; CEZANNE	◆	
46	80124	VCPIP1	DUBA3; VCIP135; FLJ23132; FLJ60694; KIAA1850; DKFZp686G038	◆	
47	7128	TNFAIP3	A20; OTUD7C; TNFA1P2; MGC104522; MGC138687; MGC138688	◆	■
48	79184	BRCC3	C6.1A; BRCC36; CXorf53; RP11-143H17.2	◆	■
49	10987	COPS5	CSN5; JAB1; SGN5; MOV-34; MGC3149	◆	■
50	10980	COPS6	CSN6; MOV34-34KD	◆	■
51	8667	EIF3H	EIF3S3; eIF3-p40; MGC102958; eIF3-gamma	◆	■
52	8665	EIF3F	EIF3S5; eIF3-p47	◆	■
53	5713	PSMD7	P40; S12; Rpn8; MOV34	◆	■
54	10213	PSMD14	PAD1; POH1; RPN11	◆	
55	10617	STAMBP	AMSH; MGC126516; MGC126518	◆	
56	57559	STAMBPL1	AMSH-FP; AMSH-LP; ALMalpha; FLJ31524; KIAA1373; bA399O19.2	◆	
57	92552	ATXN3L	MJDL; FLJ59638; MGC168806; MGC168807	◆	
58	9929	JOSD1	KIAA0063; dJ508I15.2	◆	
59	126119	JOSD2	SBBI54; FLJ29018	◆	
60		FLJ14891		◆	

The names and ID of the 60 human deubiquitinating proteins involved in this experiment. Some of the constrnct are only available in bait as indicated: ◆: Bait, ■: Prey, The clone collection is not a complete set, as the human genome encodes approximately 95 DUBs (Nijman *et al*., 2005)

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