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Title	**Minimal requirements for ubiquitination mediated regulation of thyroid hormone activation**
Authors	Péter Egri[1,2] and Balázs Gereben[1]
Affiliations	[1]Institute of Experimental Medicine, Hungarian Academy of Sciences, Dept. of Endocrine Neurobiology; Budapest, H-1083 Hungary; [2]Semmelweis University, János Szentágothai PhD School of Neurosciences, Budapest, H-1085 Hungary
Corresponding author:	Dr. Balázs Gereben, Institute of Experimental Medicine, Hungarian Academy of Sciences, Szigony str.43, Budapest, H-1083 Hungary gereben.balazs@koki.mta.hu
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Abstract

Activation of thyroxine by outer ring deiodination is the crucial first step of thyroid hormone action. Substrate-induced ubiquitination of type 2 deiodinase (D2) is the most rapid and sensitive mechanism known to regulate thyroid hormone activation. While the molecular machinery responsible for D2 ubiquitination has been extensively studied, the combination of molecular features sufficient and required to allow D2 ubiquitination remained to be determined. To address this question we constructed chimeric deiodinases by introducing different combinations of D2-specific elements into type 1 deiodinase (D1), another member of the deiodinase enzyme family, which however does not undergo ubiquitination in its native form. Studies on the chimeric proteins expressed transiently in HEK-293T cells revealed that combined insertion of the D2-specific instability loop and the K237/K244 D2 ubiquitin-carrier lysines into the corresponding positions of D1 could-not ubiquitinate D1 unless the chimera was directed to the endoplasmic reticulum (ER). Fluorescence resonance energy transfer measurements demonstrated that the C-terminal globular domain of the ER-directed chimera was able to interact with the E3 ligase subunit WSB1. However, this interaction did not occur between the chimera and the TEB4 E3 ligase although a native D2 could readily interact with the N-terminus of TEB4. In conclusion, insertion of the instability loop and ubiquitin-carrier lysines in combination with direction to the ER are sufficient and required to govern WSB1-mediated ubiquitination of an activating deiodinase enzyme.

Introduction

Thyroid hormone activation, the first step of thyroid hormone action is catalyzed by activating deiodinases, type 1 and type 2 deiodinases (D1 and D2, respectively) via outer ring deiodination (Gereben, et al. 2008). In a physiological setting, D2 is the major activating deiodinase (Maia, et al. 2005; Schneider, et al. 2006) and its activity is subjected to complex controls which also involves ubiquitination of the D2 protein (Gereben, et al. 2000).

Selective proteolysis is driven by the ubiquitin-proteasome system (UPS) and represents a crucial regulatory mechanism of cell function (Hershko and Ciechanover 1998). Targeting proteins into the proteasome for degradation is one of the most heavily studied phenomenon among the diverse set of ubiquitination-controlled cellular functions. Ubiquitination is a three-step process involving ubiquitin activation by the E1 enzyme, conjugation of ubiquitin to E2 followed by specific protein targeting by the E3 ubiquitin ligase complex responsible for substrate-recognition and specificity (Hershko and Ciechanover 1998). Proteins carrying degradative ubiquitin signal (e.g. lysine 48-linked polyubiquitin chain) are transferred into the proteasome to be cleaved to short oligopeptides or amino acids.

D2 is intrinsically unstable and is degraded in the 26S proteasome (Steinsapir, et al. 1998). D2 was identified as the first endoplasmic reticulum resident enzyme undergoing substrate-induced ubiquitination (Gereben et al. 2000). The ubiquitination of the D2 protein also involves a degradation-independent mechanism via ubiquitination induced conformational changes of D2-homodimers that results in quick decline of D2 activity (Sagar, et al. 2007). As a consequence, ubiquitination is currently considered the most rapid and efficient way to regulate D2-mediated T3 generation (Bianco and Larsen 2005).

Specific molecular elements were shown to be important for D2 ubiquitination, e.g. an instability loop between aa. 92-97 and the K237/K244 lysines in the human D2 protein (Dentice, et al. 2005; Sagar et al. 2007; Zeold, et al. 2006b). Presently, two E3 ligases are known that contribute to D2 degradation. First the SOCS-box-containing WD-40 protein (WSB1), a sonic

hedgehog-induced protein was recognized as a D2-inteacting substrate-recognition subunit of an E3 ligase catalytic core complex (ECS^{WSB1}) that consists of Elongin BC-Cullin5-Rbx1 subunits (Dentice et al. 2005). Next TEB4, the mammalian orthologue of yeast Doa10 was identified as a functional E3 ligase for the ubiquitination of D2 (Zavacki, et al. 2009) but molecular details of the TEB4-D2 interaction remains to be determined.

In contrast to D2, D1 is a long-lived plasma membrane located activating deiodinase that is not subjected to ubiquitination (Baqui, et al. 2000; Gereben et al. 2000).

Despite accumulating data, the minimal requirements of ubiquitination-mediated deiodinase regulation have not yet been defined. We aimed to determine the combination of molecular features required and sufficient to allow an activating deiodinase to be targeted by E3 ubiquitin ligases, a pre-requisite for proteins processed along the ubiquitin-proteasome pathway. We inserted D2-specific molecular elements into D1 to generate chimeric proteins that allow assessing the power of specific D2-ubiquitinating elements in the context of a natively non-ubiquitinated deiodinase protein.

Materials and Methods

Generation of DNA constructs

FLAG-tagged chimeric deiodinases were constructed using standard recombinant DNA techniques. Site-directed mutagenesis was performed with Vent polymerase PCR on templates containing the human D2 or rat D1 coding region with a cysteine-mutant active-center followed by cloning into a D10 expression vector (Gossen and Bujard 1992). For FRET experiments the generated fragments were subcloned into pEYFP-N1 (Clontech, Mountain View USA) resulting EYFP-fusion to the C-terminus of chimeras.

The human TEB4 coding region was amplified with Vent polymerase on a pcDNA3.1-GFP-TEB4 template (kind gift of Dr. M. Hochstrasser, Yale University). The product was subcloned into pEYFP-N1, pEYFP-C1 and pECFP-C1 fusion vectors (Clontech) that resulted in TEB4-Y (in pEYFP-N1), Y-TEB4 (in pEYFP-C1) and C-TEB4 (in pECFP-C1). C or Y stands for ECFP or EYFP, respectively. Construct nomenclature also provides information on the position of the fluorescent protein in the translated fusion protein (e.g. TEB4-Y indicates that EYFP was fused to the C-terminus of TEB4 protein, while the C-TEB4 construct containes ECFP at the N-terminus of TEB4). All constructs were confirmed by sequencing. The D2-Y, D2-C and C-D2 constructs (Vivek Sagar, et al. 2007) and the WSB1-C and Sec62-D1 constructs were described earlier (Vivek Sagar et al. 2007; Zeold et al. 2006b).

Cell culture and transfection

HEK-293T (Zeold, et al. 2006a) cells were grown in DMEM (Gibco, New York USA) supplemented with 10% Fetal Bovine Serum (Gibco, New York USA) and 1% penicillin-streptomycin solution (Sigma, St. Louis, USA). One day before transfection cells were plated into 35 mm-dish at 2×10^5 cells/hole concentration. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen, New York USA) following the manufacturers instruction. Secreted alkaline phosphatase (SEAP) was used as transfection internal control. 48-

hours after transfection cells were harvested and processed for Western blot or deiodinase activity measurement.

Reagents and treatments

Tetracycline (Sigma, New York USA) treatment (6- and 12-hours, 1 mg l^{-1} final concentration) was performed on the second day after transfection (Gossen and Bujard 1992). MG132 (Calbiochem, Darmstadt Germany), a protease inhibitor for proteasome activity was dissolved in DMSO and cells were treated 4 hours using 2 µmol l^{-1} final concentration versus vehicle. Hormone-free medium for T4-treatment was prepared with charcoal-stripped FBS. In brief, 100 mg charcoal (Sigma, New York USA) and 50 mg dextran (Sigma, New York USA) were preincubated overnight in 0.01 mol l^{-1} Tris buffer (pH=7.6). After centrifugation 40 ml FBS was added and incubated for 1 hour. The suspension was recentrifugated and supernatant was added to DMEM in 1:10 followed by membrane-filtration. 1 mmol l^{-1} T4 (Sigma, New York USA) stock solution was kept in NaOH.

SEAP assay

Media was removed from cells before treatment and processed for SEAP measurement using NovaBright™ Chemiluminescent SEAP Reporter Gene Assays (Invitrogen, New York USA) followed by measurement with Luminoscan Ascent (Thermo, Waltham USA) according to the manufacturer's instructions.

Western blot

Western blots were performed as described (Gereben et al. 2000). For the detection of high molecular weight ubiquitinated bands samples were run in 4-20% gradient gels (Bio-Rad, Hercules USA). The blots were incubated with M2 anti-FLAG monoclonal antibody (Sigma, New York USA) using 1:3000 dilution.

Deiodinase activity assay

HEK-293T cells were processed for *in vitro* deiodinase assay to measure the activity of D1 containing a cysteine-mutant active center according to the American Thyroid Association Guide to investigating thyroid hormone economy and action in rodent and cell models (Bianco, et al. 2014). Basic assay procedure was as previously described (Curcio-Morelli, et al. 2003). In the present study ^{125}I-T4 and 1 μmol l^{-1} T4 substrate was used for 3 hours.

Fluorescence resonance energy transfer (FRET) and confocal microscopy

Transfection was carried out as described above but the cells were plated into 35-mm glass-bottom dishes (MatTek Co, Ashland USA). FRET was performed according to our current protocol (Arrojo, et al. 2013). FRET measurement was performed on the second day after transfection using acceptor-photobleaching on a Nikon A1R laser scanning confocal system in spectral detector mode equipped with Tokai Hit stage top incubator and Supertech temperature controller. Following parameters were applied in FRET experiments: 457 nm argon-laser for ECFP excitation and 464-500 nm range for detection; 514 nm argon lasers for EYFP excitation and 516-540 nm range for detection. Cells at least 80% bleach efficiency (decrease in EYFP intensity) were involved into analysis. At least 20 cells were measured per groups.

Calculation of FRET efficiency was based on the increase of the CFP donor signal after photobleaching the YFP acceptor using the following equation:

FRET=(CFP(postbleach)-CFP(prebleach))/CFP(postbleach).

Data normalization was performed by expressing FRET efficiency of specific FRET pairs as a percentage of that of the ECFP-EYFP (C-Y) tandem construct (Cicchetti, et al. 2004). CFP and YFP (C and Y) monomers were used to determine background.

Statistical analysis

Deiodinase activities of MG132 treated samples were analyzed by t-test. FRET results were analyzed by t-test or one-way ANOVA followed by the Tukey's post-hoc test.

Results

Specific D2 elements with known effect on D2 ubiquitination were introduced into rat D1 in homologue positions. The K237/K244 ubiquitin binding lysines of D2 are phylogenetically conserved but these residues are absent in D1 proteins (**Fig.1A**). The K237 and K244 lysine residues of D2 were inserted into rat D1 by generating R223K and P230K D1 mutants, respectively. The instability loop of D2 (aa. 92-97 of human D2) was inserted between aa. 102-103 of D1 (**Fig.1A**). Direction of the chimera to stable retention in the endoplasmic reticulum was achieved by deleting its N-terminal 33 amino acids to remove the transmembrane-domain and the resulting fragment was fused to the C-terminus of human Sec62, an ER resident protein using our previously described approach (Zeold et al. 2006b). The constructs are depicted in **Fig.1B**.

The half-life of chimeras was tested in HEK-293T cells using the Tet-off expression system that allows transcriptional suppression of the transfected constructs (**Fig.2A**) (Gossen and Bujard 1992). Insertion of the ubiquitin-carrier lysines into D1 (D1-K and D1-2K) did not result in detectable changes in protein half-life and high-molecular weight ubiquitinated bands could not be observed on Western-blot (**Fig.2B**). Insertion of the D2-specific 6 aa.-loop into D1-K and D1-2K (D1-K-loop and D1-2K-loop) remarkably destabilized the chimera but ubiquitinated forms did not appear. Importantly, direction of the D1-2K-loop chimera into the ER via fusion to Sec62 (Sec62-D1-2K-loop) resulted both in destabilization and generation of high molecular weight ubiquitinated forms (**Fig.2B**).

Proteasomal uptake of the chimeric proteins was also studied by treating the cultures with 2 μmol l^{-1} MG132 for 4 hours followed by the measurement of deiodinase activity. While activity of the D2 control was readily increased, the D1-K; D1-2K; D1-K-loop and D1-2K-loop chimeras were not sensitive to MG132 similarly to native D1 indicating that these proteins are not processed by the proteasome (**Fig.2C**).

To test whether the generated chimeric proteins can bind the WSB1 E3 ligase subunit or the TEB4 E3 ligase, FRET was performed (**Fig.3A**). While the D2-binding domain of WSB1 has already been resolved (Vivek Sagar et al. 2007), localization of the substrate recognition surface of TEB4 has not been described yet. Therefore first we aimed to identify the D2-binding domain of TEB4. FRET-pairs were constructed fusing EYFP either to N- or C-terminus of TEB4 and tested in the presence of the ECFP-tagged D2 protein. Since membrane topology and localization of the TEB4 C-terminal portion is controversial (Hassink, et al. 2005; Kreft, et al. 2006) we tested both possibilities, i.e. if the C-terminus would be located either the ER-lumen or in the cytosol. Therefore we measured the interaction of the C-terminus of TEB4 (TEB4-Y) with both the C- and N-terminus of D2 (D2-C and C-D2) which are localized in the cytosol and ER-lumen, respectively (**Fig.3B,E**). Energy transfer could be detected between the N-terminus of TEB4 and the C-terminus of D2 (Y-TEB4 *vs.* D2-C) (20.04 ± 2.88 %). The strength of the interaction reached ~37 % of that of the D2-D2 homodimers (D2-C *vs.* D2-Y) (53.71 ± 1.45 %) (**Fig.3C**). In contrast, no energy transfer occurred when the C-terminus of TEB4 was tested either in the presence of the cytosolic C-terminus (TEB4-Y *vs.* D2-C) or the ER-lumen localized N-terminus (TEB4-Y *vs.* C-D2) of D2 (0.73 ± 1.78 %) (**Fig.3C**). T4, the substrate known to accelerate D2 ubiquitination increased the strength of the interaction between the N-terminus of TEB4 and C-terminus of D2 (Y-TEB4 *vs.* D2-C) by ~40% after 4 h incubation with 10 µM T4 in hormone-free media. However, no significant change occurred when the C-terminus of TEB4 (TEB4-Y) was tested although the FRET signal of the D2-D2 homodimer control decreased, as expected (**Fig.3D**).

We then moved to test the binding of the chimeras to the WSB1 E3 ligase subunit and to the TEB4 E3 ligase. The chimeras were tagged by EYFP on the C-terminus and energy transfer was measured in the presence of WSB1 (tagged on the C-terminus with ECFP, WSB1-C) or TEB4 (ECFP on its N-terminus, C-TEB4) following the scheme depicted on **Fig.4A**. Inserting the ubiquitin-carrier lysine residues of D2 into D1 by R223K and P230K mutations in the combination with the 6 aa.-loop (D1-2K-loop-Y) did not result in detectable interaction between the chimeras and WSB1-C or C-TEB4 and performed similarly to native, or ER-inserted D1 (Sec62-D1-Y) (**Fig.4B,C**). Interestingly the ER-localized lysines- and 6 aa-loop-containing mutant

206 (Sec62-D1-2K-loop-Y) showed measurable FRET signal in pair with WSB1-C (53.14 ± 14.63 %) but
207 not with C-TEB4 (**Fig.4B,D**).

Discussion

Thyroid hormone metabolism catalyzed by selenodeiodinase enzymes allows the accurate and flexible regulation of tissue-specific thyroid hormone levels (Gereben et al. 2008). Although both D1 and D2 are capable to generate T3 from thyroxin, under physiological conditions D2 is the major T3 producer due to its high substrate affinity (Maia et al. 2005). D2 is subjected to tight and multilevel control (Gereben et al. 2008) that includes substrate-mediated ubiquitination, a process representing the fastest and most sensitive regulation of thyroid hormone activation. The efficiency of this process is explained by the complex nature of D2 ubiquitination, that occurs not only via the degradation of the D2 protein, but also involves D2 inactivation in degradation-independent manner due to ubiquitination-induced conformational changes of the globular domain of the D2 homodimers allowing fast and reversible control of D2 activity (Sagar et al. 2007). This is especially useful for the cell taking into account both the energy-consuming multistep process required to incorporate the specific amino acid selenocysteine into the D2 protein and the need for rapid regulation of D2-mediated T3 generation. In contrast, the D1 protein is not processed along this pathway and represents stability (Gereben et al. 2000). Subcellular localization of the two enzymes is also different since D2 is subjected to stable retention in the endoplasmic reticulum while D1 is located in the plasma membrane (Baqui et al. 2000; Zeold et al. 2006a).

Previous studies identified molecular elements involved in the maintenance of metabolic instability of the D2 molecule (Dentice et al. 2005; Zeold et al. 2006a). However, the combination of elements required and sufficient for D2 ubiquitination has not been resolved. In the present study we used a reversed strategy by incorporating D2-specific elements into the long-lived deiodinase D1 in order to test the power of specific molecular combinations to destabilize an otherwise non-ubiquitinated protein.

Insertion of the ubiquitin-binding K237/K244 lysines into homologous positions of D1 (D1-K and D1-2K) were not sufficient to destabilize or ubiquitinate the chimera. Interestingly,

insertion of the lysines in combination with the D2 instability loop (D1-loop-K and D1-loop-2K) decreased stability without the appearance of detectable amount of ubiquitinated forms and remained insensitive to MG132 proving that were not subjected to proteasomal uptake. This observation raised the possibility that the inherent instability of D2 is not exclusively dependent on the ubiquitin-proteasome system but might be also affected by other, proteasome-independent mechanisms, as shown for iron regulatory protein 2 and IKBA (Chang, et al. 2011; Shumway and Miyamoto 2004). However, Sec62-mediated direction of the loop- and lysines-containing D1 into the ER resulted intense ubiquitination of the chimera indicating that this combination is sufficient to drive the chimera to the ubiquitin-proteasome pathway. The Sec62-fused deiodinases are inactive due to the lacking transmembrane domain thus their activity cannot be tested (Zeold et al. 2006a).

Since ubiquitin-ligase binding is crucial for the substrate-specificity of ubiquitination we studied whether the chimeric proteins could be recognized by the D2-specific E3 ligases WSB1 and TEB4 in live HEK-293T cells. WSB1 is a WD40 repeat and SOCS-box containing protein (Hilton, et al. 1998). It was demonstrated that WSB1 works as a substrate-recognizing subunit of a ECS^{WSB1} E3 ligase complex and mediates substrate induced ubiquitination of D2 (Dentice et al. 2005) binding the C-terminus of D2 via its SOCS-box domain (Sagar et al. 2007). However the recognition of D2 by TEB4 is less understood. Therefore first we had to identify the D2 interacting domain of TEB4 and showed that its N-terminus is responsible for this action. Importantly, this region contains the catalytically active RING domain that represents a common structural unit in an E3 ligase subclass (Deshaies and Joazeiro 2009). Our data demonstrate that the TEB4 protein integrates the ability of recognition and ubiquitination of D2 and its interaction with D2 is increased upon T4 exposure that results in substrate-mediated down-regulation of D2 activity.

Having identified the basic topology of the D2-TEB4 interaction we used this information to perform FRET studies on the interaction between the chimeras and TEB4 and also WSB1. We obtained evidence that the ubiquitination of D2 lysine- and loop-containing ER-localized chimera (Sec62-D1-2K-loop) binds WSB1. This demonstrates that the ubiquitination of the Sec62-D1-2K-loop protein carried out by an E3 ligase driven specific process rather than an

ERAD-driven clearance mechanism of the exogenous protein. Lack of WSB1 binding of the D1-2K-loop chimera is in accordance with the lack of ubiquitinated forms observed when studying this chimera by Western blot. Therefore we conclude that insertion of the instability loop and ubiquitin-carrier lysines into an ER located activating deiodinase are sufficient and required to govern WSB1 mediated ubiquitination. We could not detect interaction between TEB4 and the Sec62 fused chimera, although the fusion allowed directing the chimera into the ER-linked location of native D2, and the C -terminus D2 was able to interact with the N-terminus of Teb4 (Fig 3C). These findings indicate that the lack of interaction between the chimera and Teb4 cannot be explained by topology and Teb4 binding would require still unidentified molecular elements in the deiodinase protein.

It has been shown that D2 undergoes classical K48-linked ubiquitination targeting the protein for proteasomal degradation (Arrojo et al. 2013). While data have been accumulating on the biological significance of unconventional polyubiquitin-chains not composed by K48-linked ubiquitin (Kulathu and Komander 2012), presently no data are available on such alternatively linked ubiquitin on the D2 protein. Consequently, it is also unknown whether D2-specific E3 ligases would be differently involved in the generation of alternatively-linked ubiquitin chains, e.g. via the formation of non-proteasomal signals. These signals could be relevant for D2, since beyond proteasomal degradation D2 ubiquitination also drives proteasome independent conformational changes of the D2 homodimers, resulting in transient loss of D2 activity (Sagar et al. 2007). It has been also demonstrated that Doa10 (yeast orthologue of mammalian TEB4), in combination with the Ubc6 (yeast orthologue of mammalian UBE2J) ubiquitin conjugating E2 enzyme can be potentially involved in the K11-linked polyubiquitin-chain synthesis (Xu, et al. 2009). Since UBE2J was also shown to be involved in the ubiquitination of D2 (Botero, et al. 2002; Kim, et al. 2003) these data raise the possibility that TEB4 could potentially facilitate K11-linked polyubiquitin-chain formation on D2.

In conclusion, the obtained data identified a combined set of molecular elements and intracellular localization necessary for WSB1 mediated regulation of thyroid hormone activation and demonstrated distinct requirements for WSB1 and TEB4-mediated ubiquitination of D2. *In vivo*, the complex regulation of deiodination via WSB1 and TEB4 could play a role in D2

295 expressing tanycytes of the mediobasal hypothalamus known to coexpress WSB1, TEB4 and
296 deubiquitinase enzymes (Fekete, et al. 2007; Zavacki et al. 2009).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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Figure legends

Figure 1.

(A) Alignment of amino acid sequences of D1 and D2 portions in different species. Amino acid positions indicated using the positons of amino acids in human D2, the first six amino acids of the D2 specific instability loop and the conserved lysine residues were boxed. Arrows indicate the position of ubiquitin-carrier lysines of D2. **(B)** Schematic depiction of the applied D1-D2 recombinant chimeric proteins tagged with a FLAG epitope on the N-terminus.

Figure 2. Stability and ubiquitination of chimeric deiodinase proteins.

(A) Experimental design. **(B)** Assessment of protein stability using of 1 mg l^{-1} tetracycline treatment for 12h in HEK-293T cells. FLAG-tag was detected by Western-blot. Open arrows point to high molecular mass ubiquitinated deiodinase forms. The asterisk labeled inset demonstrates short exposure of Sec62-D1-2K-loop +/- tetracycline (left and right, respectively) run in 10 % SDS gel. **(C)** D1 activity of chimeras expressed in HEK-293T cells treated with 2 µmol l^{-1} MG132 for 4-hours. D2 was used as a positive control of MG132 treatment. Activity was expressed in percentage of the vehicle threated corresponding control (MG132/DMSO) normalized by SEAP (mean ± SEM; n=3) **: $p<0.01$ by t-test.

Figure 3. Topology and substrate-dependence of the D2-TEB4 interaction assessed with FRET

(A) Experimental design. **(B)** Schematic depiction of FRET-pairs. Due to the controversial topology of the C-terminus of TEB4 both cytosolic and ER lumen located positions were depicted **(C)** FRET-assisted detection of the topology of TEB4-D2 interaction. Data are expressed as a percentage of that of the fused ECFP-EYFP (C-Y) tandem positive control while cotransfected monomeric ECFP (C) and EYFP (Y) were applied to detect non-specific background (mean ± SEM; n≥15 per group) ***: $p<0.001$; **: $p<0.01$ by one-way ANOVA *vs.* monomer followed by Tukey's post-hoc test). **(D)** Effect of 1 µmol l^{-1} T4 on the D2-TEB4 interaction. T4

sensitive D2 homodimers were used as positive controls (mean ± SEM; n≥15 per group) ***: p<0.001; **: p<0.01 by two-tailed t-test. **(E)** Photomicrography of individual HEK-293T cells demonstrating acceptor photobleaching FRET to detect the interaction between TEB4 and D2. Left-top: prebleach (pre) acceptor; right-top: postbleach (post) acceptor; left-bottom: prebleached donor; right-bottom: postbleached donor. The order of the fluorescent protein (C or Y) and the tagged protein in the name of the constructs reflects their position in the fusion protein.

Figure 4. Interaction of deiodinase chimeras with the WSB1 and TEB4 E3 ligases assessed with FRET

(A) Experimental design. **(B)** FRET efficiency of chimeric deiodinases coexpressed with WSB1-C or C-TEB4 in HEK-293T cells. Data are expressed as a percentage of that of the fused CFP-YFP (C-Y) tandem positive control while cotransfected monomeric CFP (C) and YFP (Y) were applied to detect non-specific background (mean ± SEM; n≥30 per group) ***: p<0.001; **: p<0.01 One-way ANOVA *vs.* monomer followed by Tukey's post-hoc test. **(C)** Photomicrography of individual HEK-293T cells demonstrating FRET between D1-D2 chimeric proteins and WSB1. **(D)** Same as (C) with TEB4. Each panel contains the following order of pictures: left-top: prebleach (pre) acceptor; right-top: postbleach (post) acceptor; left-bottom: prebleached donor; right-bottom: postbleached donor. The order of the fluorescent protein (C or Y) and the tagged protein in the name of the constructs reflects their position in the fusion protein.

Figure 1

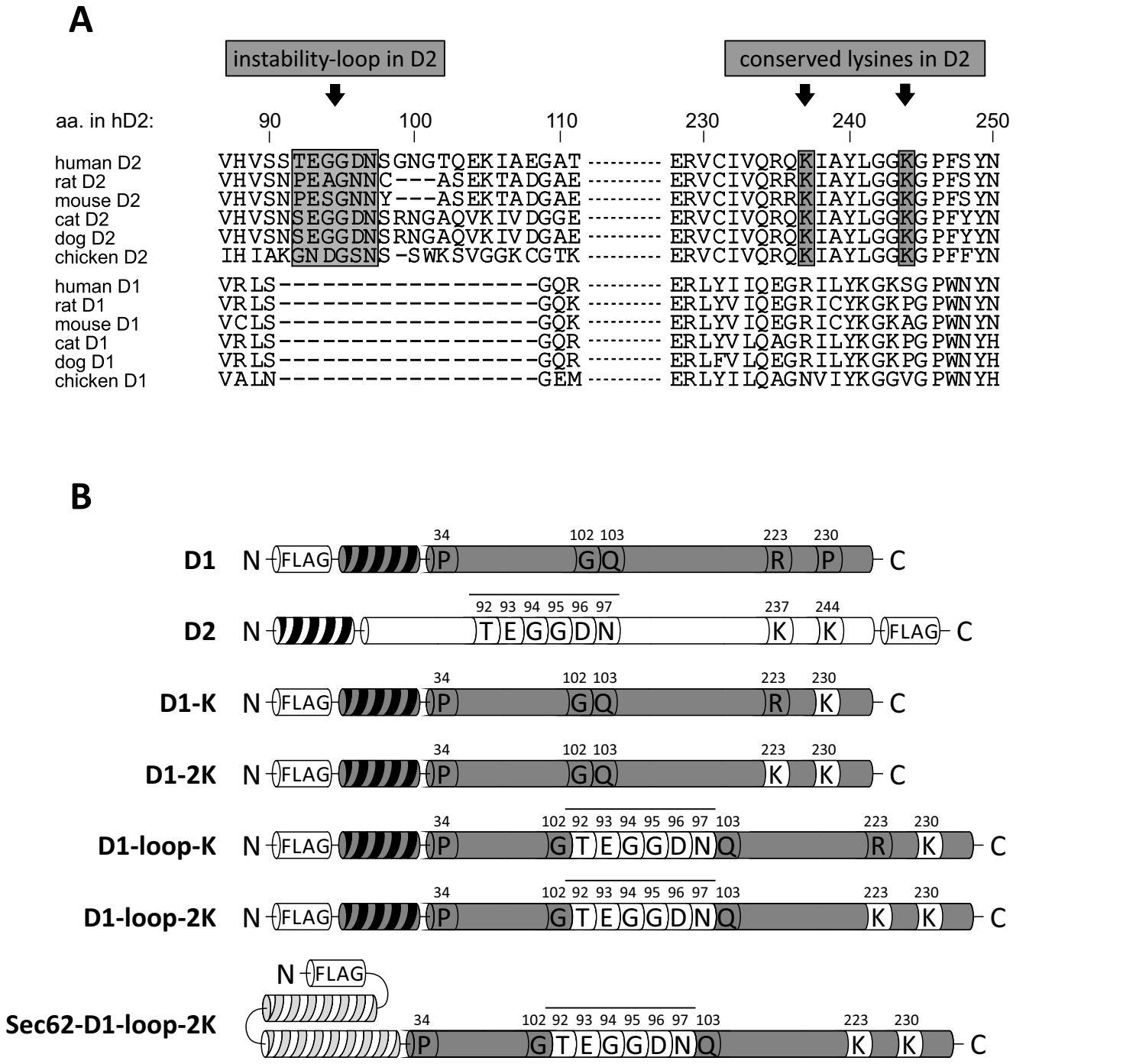

A
instability-loop in D2
conserved lysines in D2
aa. in hD2:
90
100
110
230
240
250
human D2 VHVSSTEGGDNSGNGTQEKIAEGAT---------ERVCIVQRQKIAYLGGKGPFSYN
rat D2 VHVSNPEAGNNC---ASEKTADGAE---------ERVCIVQRRKIAYLGGKGPFSYN
mouse D2 VHVSNPESGNNY---ASEKTADGAE---------ERVCIVQRRKIAYLGGKGPFSYN
cat D2 VHVSNSEGGDNSRNGAQVKIVDGGE---------ERVCIVQRQKIAYLGGKGPFYYN
dog D2 VHVSNSEGGDNSRNGAQVKIVDGAE---------ERVCIVQRQKIAYLGGKGPFYYN
chicken D2 IHIAKGNDGSNS-SWKSVGGKCGTK---------ERVCIVQRQKIAYLGGKGPFFYN
human D1 VRLS----------GQR---------ERLYIIQEGRILYKGKSGPWNYN
rat D1 VRLS----------GQK---------ERLYVIQEGRICYKGKPGPWNYN
mouse D1 VCLS----------GQK---------ERLYVIQEGRICYKGKAGPWNYN
cat D1 VRLS----------GQR---------ERLYVLQAGRILYKGKPGPWNYH
dog D1 VRLS----------GQR---------ERLFVLQEGRILYKGKPGPWNYH
chicken D1 VALN----------GEM---------ERLYILQAGNVIYKGGVGPWNYH
B
D1
D2
D1-K
D1-2K
D1-loop-K
D1-loop-2K
Sec62-D1-loop-2K
N
C
FLAG
34
102 103
223
230
92 93 94 95 96 97
237
244
103

Figure 2

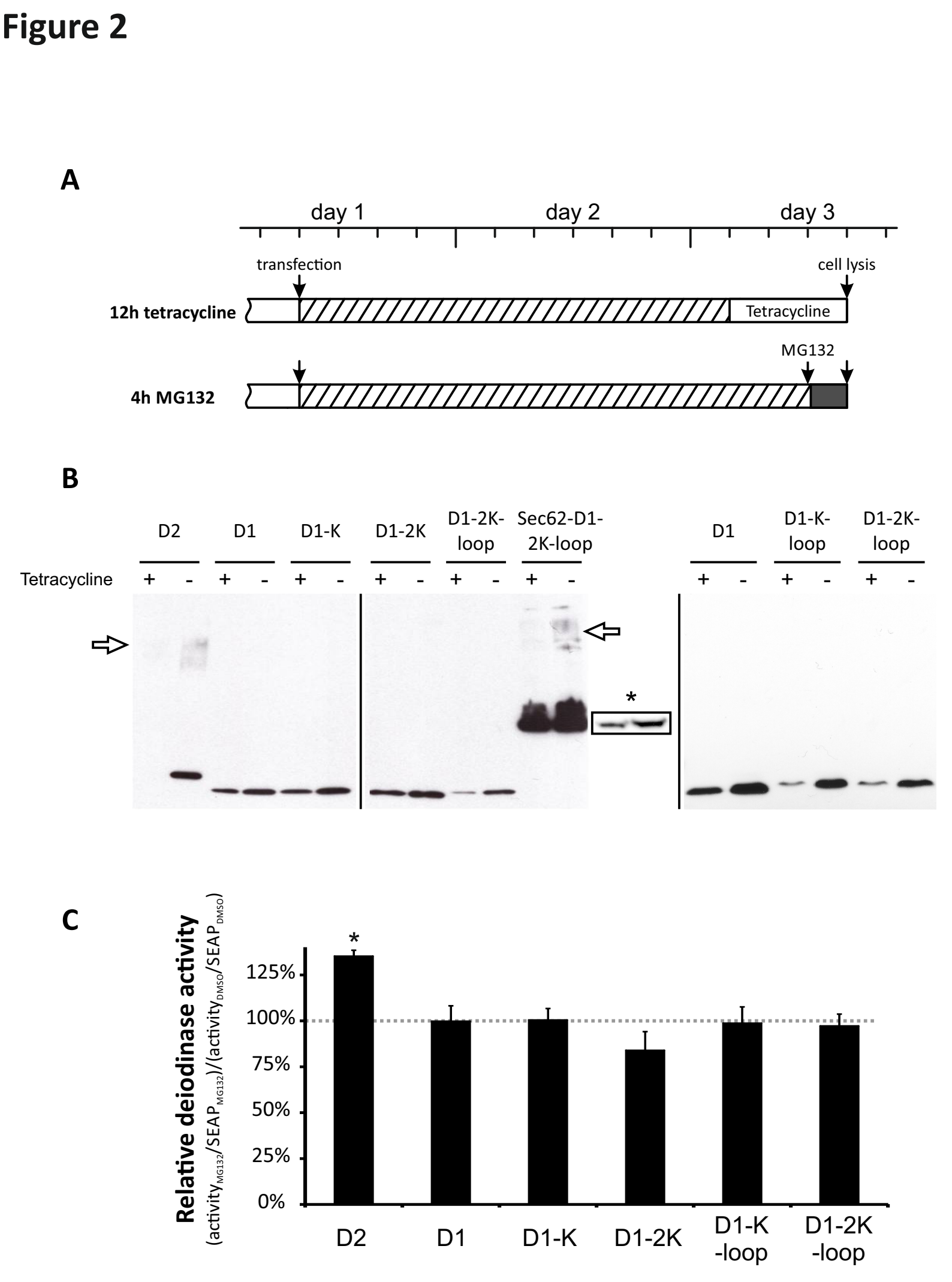
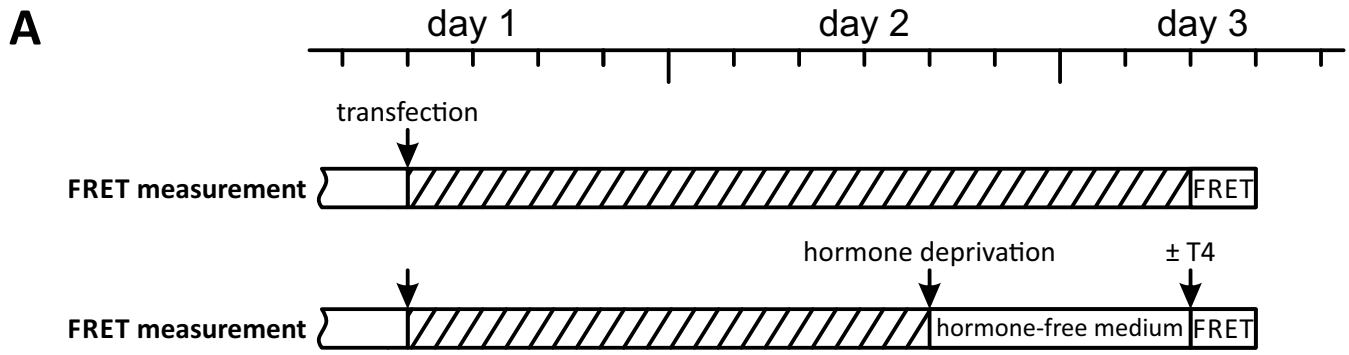
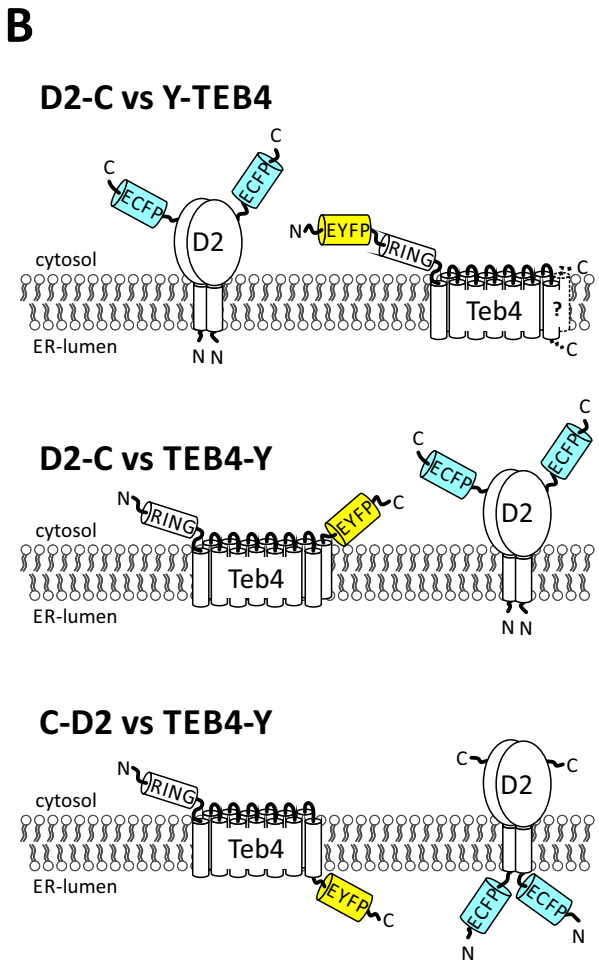
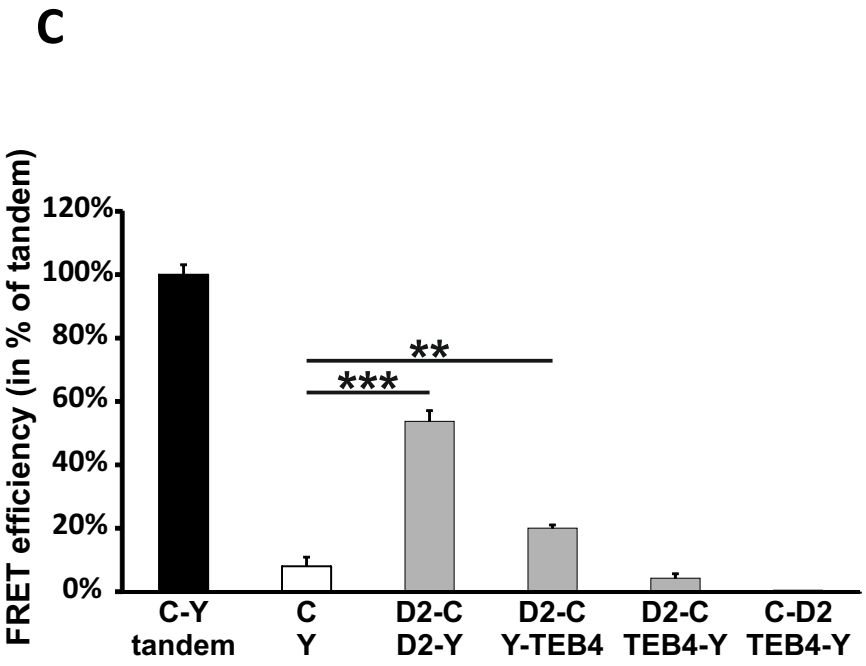
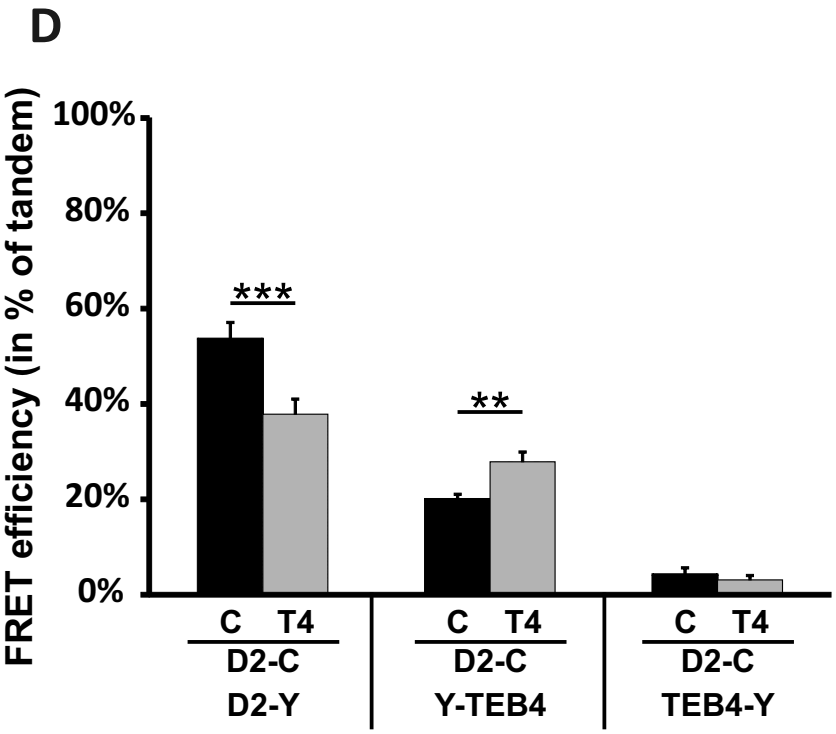

A
day 1
day 2
day 3
transfection
cell lysis
12h tetracycline
Tetracycline
MG132
4h MG132
B
D2
D1
D1-K
D1-2K
D1-2K-loop
Sec62-D1-2K-loop
D1
D1-K-loop
D1-2K-loop
Tetracycline
+ - + - + - + - + - + - + - + - + -
*
C
Relative deiodinase activity
(activity$_{MG132}$/SEAP$_{MG132}$)/(activity$_{DMSO}$/SEAP$_{DMSO}$)
*
125%
100%
75%
50%
25%
0%
D2
D1
D1-K
D1-2K
D1-K -loop
D1-2K -loop

Figure 3


A
day 1
day 2
day 3
transfection
FRET measurement
FRET
hormone deprivation
± T4
FRET measurement
hormone-free medium
FRET


B
D2-C vs Y-TEB4
ECFP
D2
EYFP
VRING
cytosol
Teb4
ER-lumen
D2-C vs TEB4-Y
C-D2 vs TEB4-Y


C
FRET efficiency (in % of tandem)
120%
100%
80%
60%
40%
20%
0%
**
***
C-Y tandem
C Y
D2-C D2-Y
D2-C Y-TEB4
D2-C TEB4-Y
C-D2 TEB4-Y


D
FRET efficiency (in % of tandem)
100%
80%
60%
40%
20%
0%
***
**
C T4
D2-C D2-Y
C T4
D2-C Y-TEB4
C T4
D2-C TEB4-Y

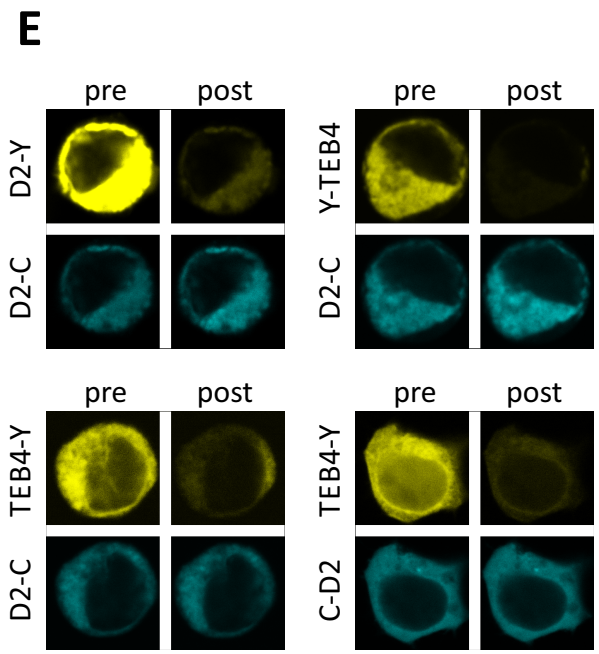
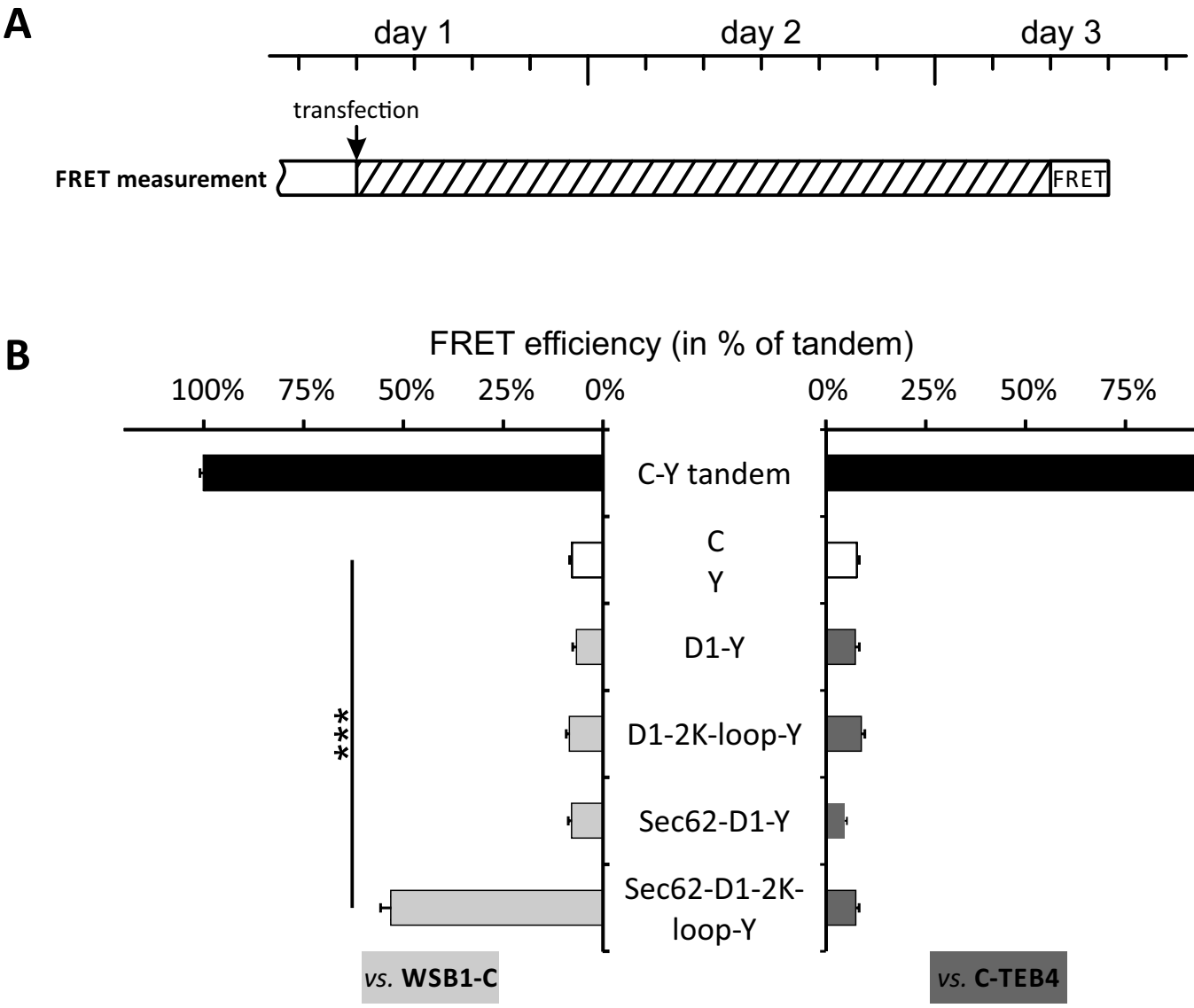

E
pre
post
D2-Y
D2-C
Y-TEB4
TEB4-Y
C-D2

Figure 4


A
day 1
day 2
day 3
transfection
FRET measurement
FRET
B
FRET efficiency (in % of tandem)
100%
75%
50%
25%
0%
0%
25%
50%
75%
100%
C-Y tandem
C
Y
D1-Y
D1-2K-loop-Y
Sec62-D1-Y
Sec62-D1-2K-loop-Y
***
vs. WSB1-C
vs. C-TEB4
C
pre
post
D1-Y
WSB1-C
D1-2K-loop-Y
Sec62-D1-Y
Sec62-D1-2K-loop-Y
D
C-TEB4