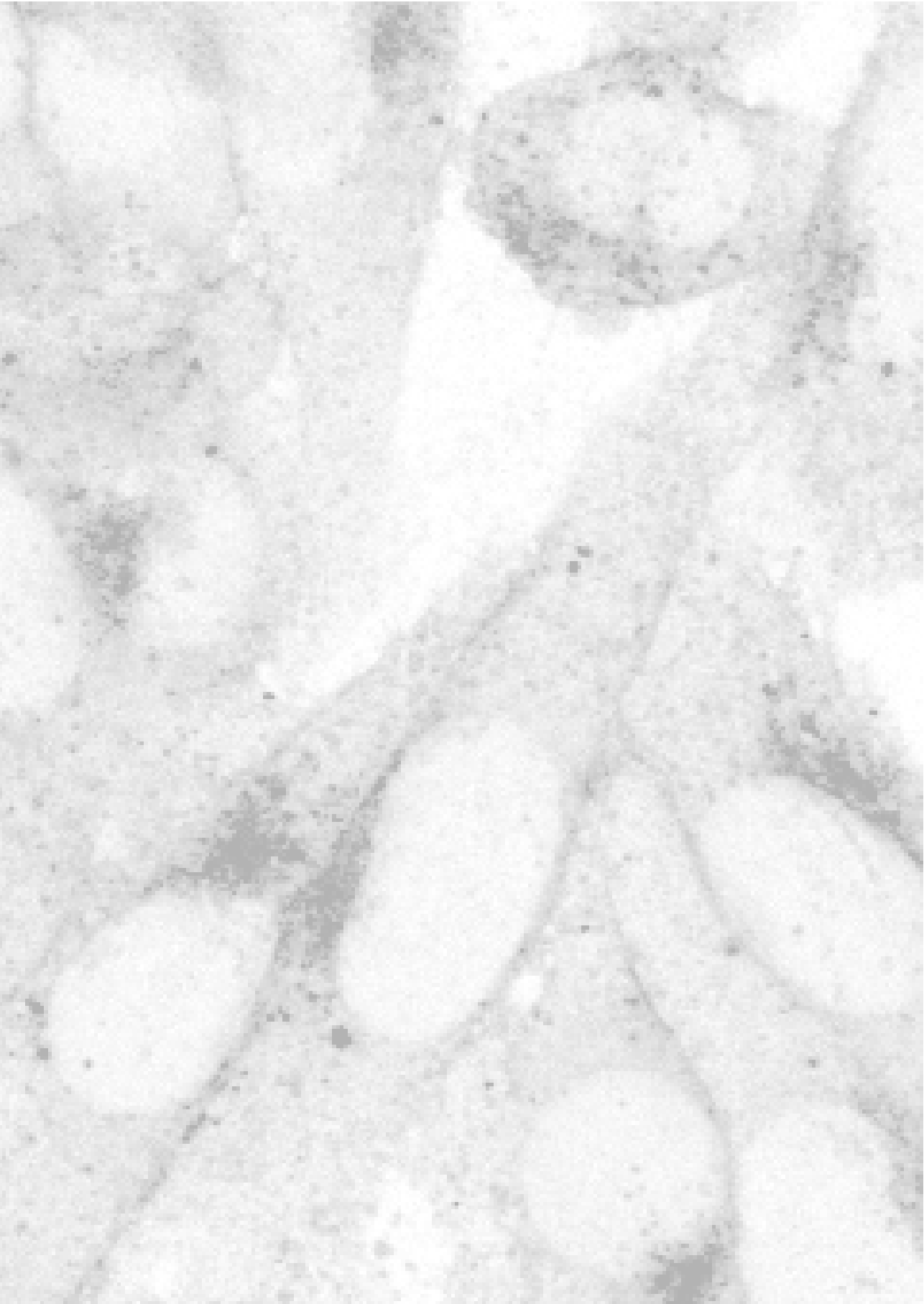

Chapter 5:
Identification of an E2 enzyme for the ER embedded E3 ligase RNF26

M.L.M. Jongsma, I. Berlin and J. Neefjes

Ubiquitination is a post-translational modification requiring E1-activating, E2-conjugating and E3-ligating enzymes. Only a few E1 enzymes are identified, while around 40 E2 enzymes and over 600 E3 ligases are known. E3 ligases can pair with multiple E2 enzymes and the bound E2 enzyme determines which ubiquitin-chain-type is added to the substrate. Since the ubiquitin-chain-type determines the fate of the target protein, it is important to identify which E2 enzymes pairs to a certain E3 ligase. Here I describe a method which identified ten potential E2 enzymes for the E3 ligase RNF26, with as most compelling candidate the integral ER membrane E2 enzyme UBE2J1.

The E3 ligase RNF26 controls endosomal positioning in interphase and mitotic cells by the actin-based motor protein Myosin VI (Chapter 4). We identified various RNF26 interacting proteins that also control endosomal positioning and are ubiquitinated by RNF26. Ubiquitination not only requires an E3 ligase, but also an E1 and E2 enzyme. To understand RNF26 regulated ubiquitination, the E2 enzyme pairing with RNF26 for activity should also be identified.

Ubiquitination of a substrate requires three steps: 1. Ubiquitin is activated by one of the few E1's (ubiquitin-activating) enzymes defined. As an energy source, ATP is used. 2. Ubiquitin is transferred from the E1 to an E2 (ubiquitin-conjugating) enzyme. 3. An E3 ligase binds both the target protein and the E2 enzyme bound to ubiquitin, which allows transfer of the ubiquitin moiety to a lysine of the target protein. This transfer can occur either directly from the E2 enzyme to the target protein (RING-domain containing E3's) or ubiquitin is first transferred to the E3 ligase before being coupled to the target protein (HECT-domain containing E3's) [1-3].

Thus far only a few E1 enzymes are known, while around 40 E2 enzymes and over 600 different E3 ligases are identified. These numbers imply that various E2 enzymes should be able to pair with multiple E3 ligases. Since HECT E3 ligases first accept the ubiquitin molecule from their E2 enzymes, they determine the type of ubiquitin-chain on the substrate independently of the interacting E2 [4]. On the other hand, RING ligases transfer ubiquitin directly from the E2 enzyme to the substrate. Therefore the interacting E2 enzyme determines the type of ubiquitin transferred to the substrate [5,6]. Such a process has been shown for the RING E3 ligase BRCA1. Ten E2 enzymes are identified to interact with BRCA1, which have been shown to possess different ubiquitin-transfer properties. While some E2 enzymes transfer polyubiquitin chains to BRCA1, other E2 enzymes transfer mono-ubiquitin moieties after which other E2 enzymes elongated the ubiquitin-chain with specific chain-linkages on mono-ubiquitinated BRCA1 [7]. Since the type of the ubiquitin-chain determines the fate of a substrate, identification of E2 enzymes for RNF26 is critical in understanding its function.

The RING domain of an E3 ligase interacts with the UBC-domains of their specific E2 enzymes. These UBC domains of E2 enzymes and the RING domains of E3 ligases are highly conserved. Nevertheless, an E3 ligase does not interact with all E2 enzymes, but only to a specific subset. There are several reasons why an E2 can or cannot interact with a certain E3 ligase. First, posttranslational modifications such as sumoylation and phosphorylation on the E2 enzyme can prevent or promote its interaction with an E3 ligase [8,9]. Second, the E2 enzyme and the E3 ligase need to have the same subcellular localization and tissue distribution. Third, besides the highly conserved E2 UBC-domain, also other less conserved motifs in the E2 molecule can modulate its interaction with an E3 ligase [10,11].

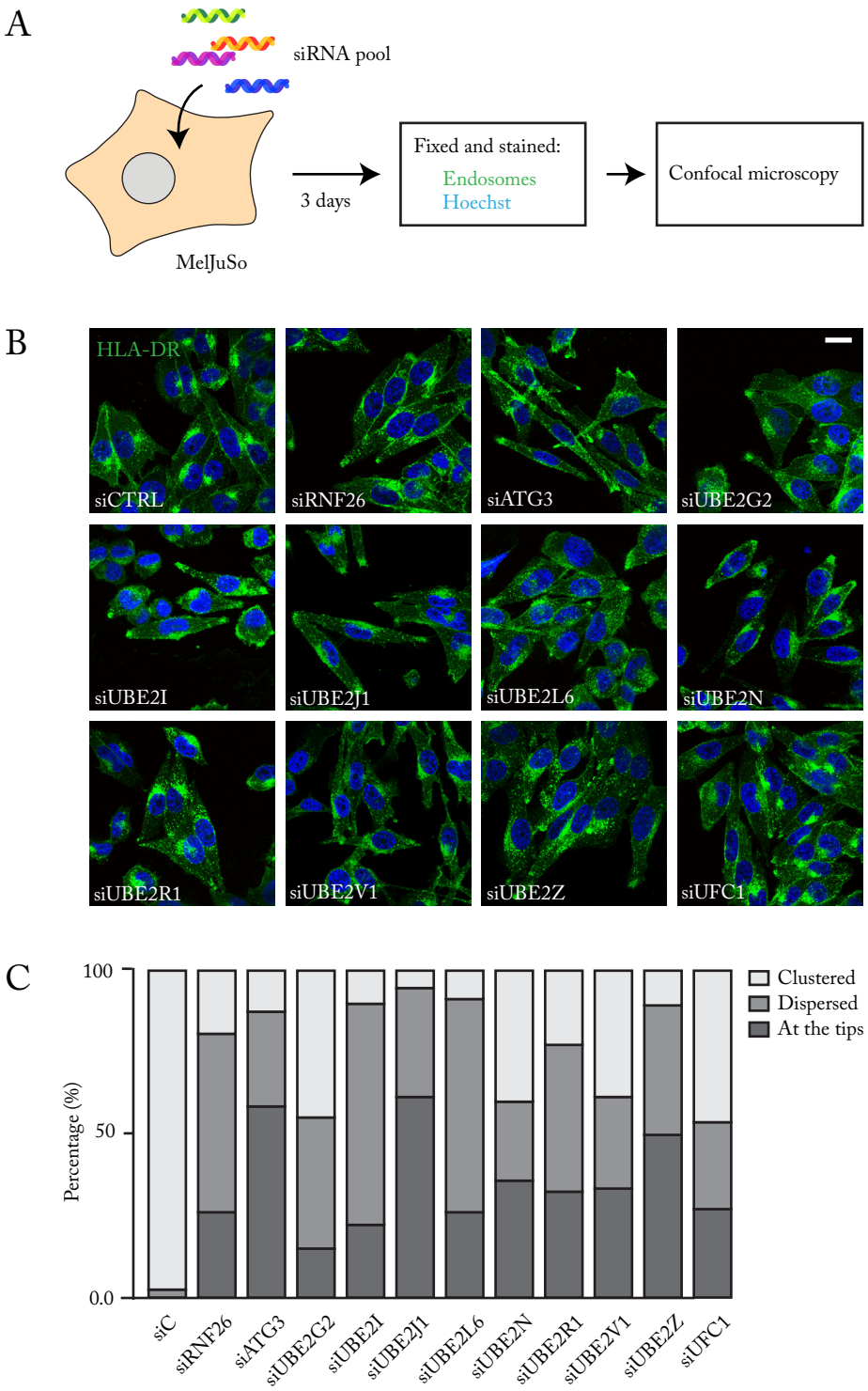
A
siRNA pool
MelJuSo
3 days
Fixed and stained:
Endosomes
Hoechst
Confocal microscopy
B
HLA-DR
siCTRL
siRNF26
siATG3
siUBE2G2
siUBE2I
siUBE2J1
siUBE2L6
siUBE2N
siUBE2R1
siUBE2V1
siUBE2Z
siUFC1
C
100
50
0.0
Percentage (%)
Clustered
Dispersed
At the tips
siC
siRNF26
siATG3
siUBE2G2
siUBE2I
siUBE2J1
siUBE2L6
siUBE2N
siUBE2R1
siUBE2V1
siUBE2Z
siUFC1

Figure 1 | **A small scale RNAi-based screen to identify RNF26 E2 enzymes.** (A) MelJuso cells were transfected with siRNA pools targeting 40 different E2 enzymes. Three days post transfection the cells were fixed and stained for the nucleus (Hoechst) and late endosomes (anti-HLA-DR). Confocal microscopy was used as a read out. (B) Depletion of ten E2 enzymes showed dispersion of late endosomes comparable to the phenotype in RNF26 depleted cells. MelJuSo cells treated with a non-targeting siRNA (siC) show normal late endosomal distribution, namely a compact cloud of late endosomes near the nucleus. (C) Quantification of the late endosomal dispersion phenotypes after E2 depletion. (Bar = 10μm)

One way to identify the E2 enzyme for an E3 ligase is by Yeast-two-hybrid. In 2009, a yeast-two-hybrid screen was performed using the catalytic domains of 35 human E2 enzymes and 250 RING-type-E3 ligases. This lead to the identification of over 300 new (and already known) E2-E3 pairs [12]. Here we describe a more functional approach to identify E2 enzymes for the E3 ligase RNF26. Since silencing of RNF26 results in dispersed endosomes, we used this phenotype as a read-out for a RNAi-based E2-screen. We identified ten E2 enzymes as potential E2s for RNF26. Based on location and biochemistry, we show that the integral ER membrane E2 enzyme UBE2J1 is the most likely E2 candidates for pairing with RNF26. UBE2J1 is an E2 enzyme previously shown to also be involved in ER associated degradation (ERAD) [13], which would then connect ERAD with the positioning of endosomal compartments by active RNF26.

Results

A small-scale siRNA-based screen identified potential RNF26 E2 enzymes

As discussed in Chapter 4 of this thesis, endosomal localization is controlled by RNF26 regulated ubiquitination events. To be able to ubiquitinate its target proteins, the E3 ligase RNF26 needs to pair with an E2 enzyme. To identify potential RNF26 binding E2 enzymes, we performed a small-scale siRNA-based screen using confocal microscopy as a read out. A pool of four siRNAs, targeting each of the 40 different known E2 enzymes were used to silence the various E2 enzymes in MelJuSo cells. Three days post-transfection, the cells were fixed and endosomes were stained with anti-HLA-DR antibodies (Figure 1A). Confocal microscopy was used to determine the effects of E2 enzyme depletion on endosome localization. Since depletion of RNF26 results in dispersion of endosomes, we selected the E2 enzymes resulting in a comparable phenotype. Depletion of ten E2 enzymes resulted in endosomal dispersion (Figure 1B; quantified in Figure 1C). General information about these ten E2 enzymes can be found in Table 2.

UBE2J1 is a likely candidate for RNF26

We have shown before that RNF26 has to be ER membrane embedded to control the location of the endosomal pathway (Chapter 4). The cytosolic RNF26 Tail domain has activity and the potential to ubiquitinate substrates (thus the Tail-domain alone does interact with E2 enzymes) but does not affect endosomal location. This suggest that the E2 enzyme interactingwith RNF26 involved in the control of endosomal vesicle localization would also be ER membrane bound. Of the ten candidate E2 enzymes, UBE2J1 fulfilled these criteria. UBE2J1 contains a transmembrane domain and is, like RNF26, localized in the ER membrane [14]. We co-expressed RFP-RNF26 and FLAG-UBE2J1 in HEK293T cells and were able to co-isolate the two complexes (Figure 2A). If RNF26 and UBE2J1 form an active E2:E3 pair, interaction between the two proteins will recruit ubiquitin for target proteins ubiquitination. To determine if RNF26 and UBE2J1 have the potential recruit ubiquitin, we overexpressed FLAG-UBE2J1 and active HA-RNF26 in MelJuSo cells and stained for endogenous ubiquitin. Using confocal-microscopy, RNF26 and UBE2J1 co-localized on many sites in the

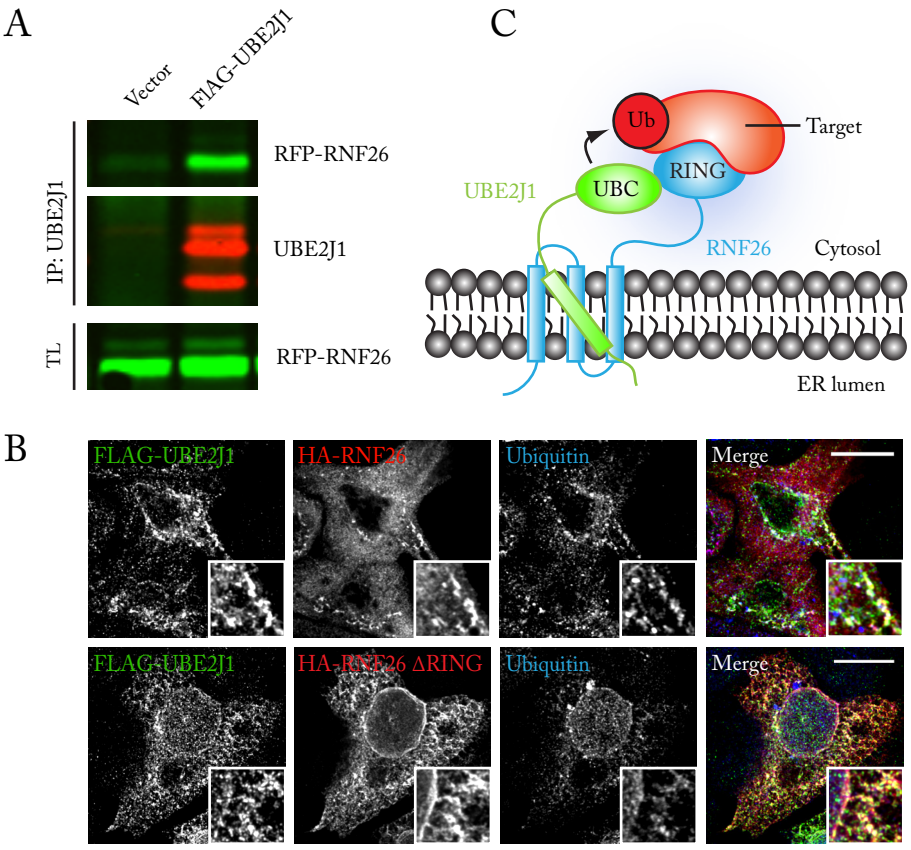

Figure 2 | **UBE2J1 is a potential E2 enzyme of RNF26.** (A) Co-immunoprecipitation of FLAG-UBE2J1 and RFP-RNF26 showed a clear interaction between the E2 enzyme and the E3 ligase. (B) A hypothetical model shows an interaction between the transmembrane domains of the E2 UBE2J1 and the E3 ligase RNF26 leading to target protein ubiquitination. (C) FLAG-UBE2J1 transiently transfected in MelJuSo cells shows co-localization with both HA-RNF26 and inactive HA-RNF26 ΔRING in the ER membrane. Ubiquitin was recruited to spots containing UBE2J1 and active RNF26, while the amount of ubiquitin recruited to areas containing both UBE2J1 and inactive RNF26 ΔRING was strongly reduced. (Bar = 10μm)

ER membrane as did ubiquitin. As a control we also located UBE2J1 together with inactive RNF26ΔRING and ubiquitin. While UBE2J1 still co-localized with RNF26 missing its RING domain, ubiquitin recruitment to the ER membrane was strongly reduced since E3 ligase activity was absent. This suggests that the ER localized E2 enzyme UBE2J1 is an attractive partner for RNF26 interacting independently of the RNF26 RING domain. Based on these results we hypothesize that the E2 enzyme UBE2J1 and the E3 ligase RNF26 interact via their transmembrane domains in the ER membrane. This interacting brings together their UBC and RING domain leading to target proteins ubiquitination (Figure 2C).

Discussion

We could identify ten candidate E2 enzymes for the E3 ligase RNF26 using a phenotypic microscopy based siRNA screen. Like RNF26, these E2 enzymes showed endosomal dispersion after their depletion in MelJuSo cells. One of the hits, the ER membrane protein UBE2J1, is a likely can-

didate because: it co-isolates with RNF26, co-localizes with RNF26 and RNF26 has to be ER bound for function as does this E2 enzyme. Whether the transmembrane segments of UBE2J1 and RNF26 interact and support a more stable association, still has to be defined.

A yeast-two-hybrid screen identified RNF26 E2 enzymes: We used RNA interference as a method to identify an E2:E3 pair. Alternatively, the E2 enzyme for an E3 ligase can be identified by Yeast-two-hybrid. In 2009, a yeast-two-hybrid screen was performed using the catalytic domains of 35 human E2 enzymes and 250 RING-type-E3 ligases. This lead to the identification of over 300 new (and already known) E2-E3 pairs [12]. In this screen, the RING domain of RNF26 was also included for testing the interaction with the different E2 enzymes. A number of potential E2 enzymes were identified, most notably the E2 enzymes UBE2D2, UBE2D3, UBE2G2, UBE2Q1, UBE2U and UBE2W. The only candidate found in our screen and the Y2H screen is UBE2G2. How can these differences in candidates be explained? Although silenced in our screen, UBE2D2, UBE2D3, UBE2Q1, UBE2U and UBE2W did not show endosomal dispersion. Although this could be due to insufficient downregulation of the respective E2 enzymes, other options are also possible. Firstly, some E2s have a strict tissue distribution. For example, UBE2U is not expressed in MelJuSo cells according to MA-analysis on MelJuSo lysates [15]. It could also be that the various E2 enzymes indeed bind the RING domain of RNF26 in solution but not when membrane bound. Another membrane bound E2 (like UBE2J1) will simply more efficiently interact with the E3 ligase embedded in the same membrane. It is also possible that the E2 enzymes simply do not confer the phenotype we are interested in but support other functions of RNF26. Finally, the various E2 enzymes may bind at low affinity in yeast two-hybrid that are irrelevant under physiological conditions.

Why are some of the E2 candidates from our screen not found in the Y2H screen? In the reported Y2H screen, only interactions between RING and UBC domains were considered. Structural studies on the interaction between E2-E3 pairs showed the importance of additional motifs beyond these conserved domains. For example, the E3 ligase c-CBL and the E2 enzyme UbcH7 interact via their UBC and RING domain but also binding of the N-terminal α-helix of UbcH7 to a region of c-CBL outside the RING domain is essential [11]. Also the E3 ligase CNOT4 and the E2 UbcH5b require additional interactions between the N-terminal α-helix of UbcH5b with the CNOT4 RING domain [10]. Furthermore, some E3 ligases only interact with their E2 enzyme if they are dimers, like BRCA1 [16]. A negative result in any screen does not exclude interactions. There are many reasons why protein interactions are abrogated under experimental conditions, as illustrated above.

E2 complex formation: Some E2 enzymes have been shown to form multi-enzyme complexes, like UBE2N with UBE2V1 and UBE2V2 [17]. Although all three E2 enzymes were expressed in MelJuSo cells, only UBE2N and UBE2V1 depletion resulted in endosomal dispersion. A complex containing UBE2N and UBE2V1 may be involved in endosome localization, while interaction of UBE2N with UBE2V2 is not. Alternatively, UBE2V2 is considered redundant when UBE2V1 is around. Also UBE2V2 may simply have been insufficiently silenced to expect a phenotype. Besides, different E2 pairs can exists with different functions. For example, UBE2N-UBE2V1 has been shown to be important in nuclear factor kB (NFkB) activation, while UBE2N-UBE2V2 plays a role in DNA repair [18].

Other functions of the E2 enzyme UBE2J1: There are far fewer E2 enzymes than E3

ligases implying that E2 enzymes are shared between various E3s and that E2 enzymes are involved in various cell biology steps. This is also the case for UBE2J1, which is known for its role in ER-associated degradation (ERAD) [13]. For example, UBE2J1 in combination with the E3 ligase HRD1 has been shown to ubiquitinate misfolded MHC class I heavy chains leading to their proteasomal degradation [19].

In summary, we present here an siRNA based method to identify E2 enzymes that pair with the E3 ligase RNF26 by a functional screen. We identified ten candidates and performed additional steps to identify the E2 enzyme UBE2J1 as a likely and relevant candidate involved in the control of endosomal positioning. Both UBE2J1 and RNF26 are ER embedded enzymes that may interact by lateral diffusion. How RNF26 recruits the E2 enzyme that is shared with the ERAD machinery, is at present unclear.

Material and methods

Cell lines: Wild-type (wt) MelJuSo cells, a human melanoma cell line, were cultured in IMDM (Gibco) supplemented with 7.5% fetal calf serum (FCS, Greiner) and Penicillin/Streptomycin (Invitrogen). Human HEK293T cells were cultured in DMEM (Gibco) supplemented with 7.5% fetal calf serum (FCS, Greiner) and Penicillin/Streptomycin (Invitrogen).

Constructs: RFP-RNF26 obtained from IMAGE:3507662, was cloned into a mRFP-C1 vector using EcoRI and BamHI. RFP-RNF26 ΔRING (aa1-352) was amplified from full length RNF26 and cloned in RFP-C1 using the restriction enzymes EcoR1 and BamHI. FLAG-UBE2J1 in pcDNA3.1 was a generous gift from P. Lehner (Cambridge).

Antibodies: Rabbit anti-human HLA-DR [20], rabbit anti-FLAG (F7425, Sigma), Rat anti-HA (3F10, Roche), mouse anti-ubiquitin (P4D1, sc-8017, Santa Cruz) and Hoechst (2 μg/ml, 33342, Invitrogen) were used to stain early HLA-DR, FLAG-tagged UBE2J1, HA-tagged RNF26, ubiquitin and the nucleus respectively, followed by secondary Alexa dye-coupled antibodies (Invitrogen) or Donkey-anti-rat CF568 (20092-1 Biotium) for detection by confocal microscopy. Mouse anti-FLAG M2 (F3165, Sigma) was used for Immune precipitation assays. Rabbit anti-mRFP [21] and mouse anti-FLAG M2 (F3165, Sigma) were used for detection on Western Blot. Secondary IRDye 800CW Goat anti-rabbit IgG (H+L) (926-32211, Li-COR) and IRDye 680LT Goat anti-mouse IgG (H+L) (926-68020, Li-COR) were used for detection using the Odyssey Classic imager (Li-Cor).

siRNA transfection: In a 24-well plate, 50μl siRNA (500 nM) was mixed with 0.75μl DharmaFECT1 (Dharmacon) diluted in 49.25 μl IMDM, incubated for 20 minutes, followed by the addition of 28,000 MelJuSo cells and culture for three days at 37°C and 5% CO2 before analysis. E2 enzymes were silenced using siRNAs from the siGenome SMARTpool library (Dharmacon). Non-targeting siRNA (siCTRL, D-001206-13-20, Dharmacon) was used as a negative control.

DNA transfections: MelJuSo cells seeded in a 12-well plate were transfected using Extremegene HP (Roche). 100μl IMDM medium was mixed with 3μl Extremegene HP and 1μg DNA. After 30min, the mix was added to the MelJuSo cells followed by a further culture for one days at 37°C and 5% CO2 before analysis. HEK293T cells seeded in a 6-well plate were transfected using PEI (Polyethylenimine, 23966, Polysciences Inc.). 100μl IMDM medium was mixed with 6μl PEI and 2μg DNA. After 30min, the mix was added to the HEK293T cells and cul-

tured for one day at 37°C and 5% CO2 before analysis.

Confocal microscopy: Cells were fixed with PBS/3.75% formaldehyde (free from acid, Merck), permeabilized with PBS/0.1% TritonX-100 (Sigma) and blocked with PBS/0.5% bovine serum albumin (BSA, Sigma). Cell were stained for LEs (anti-HLA-DR) followed by the secondary antibody Alexa-488 and a nuclear staining (Hoechst). Stained cells were analyzed by a Leica SP5 microscope with appropriate filters for fluorescence detection. Pictures were taken using a HCX PL 63x 1.32 oil objective. Hoechst was excited at λ=405nm and detected at λ=416-470nm; Alexa-488 was excited at λ=488nm and detected at λ=500-550nm; Alexa-647 was excited at λ=633nm and detected at λ=642-742nm.

Ubiquitination-assay: HEK293T cells were lysed for 30 min in lysis buffer containing 50mM Tris-HCl pH7.5, 150mM NaCl, 5mM EDTA, 0.5%TX100, freshly added 10mM N-MethylMaleimide (to inhibit cysteine proteases including DUBs; diluted in DMSO) and protease inhibitors (Roche Diagnostics, EDTA free). Supernatants were stored and frozen at -80oC, thawn and sonicated (Branson Sonifier 250, 3 pulses, Duty Cycle=50%, Output=7). After spinning (10 min at max. speed) we incubated the lysates with anti-FLAG-coupled Protein G 4 fast flow (GE Healthcare) for one hour. Beads were washed four times in lysis buffer before addition of Laemmli Sample Buffer (containing 5% β-mercaptoethanol) followed by 5 min incubation at 95°C.

SDS-PAGE and Western blotting: Samples were separated on a 10% acrylamide gel and transferred to a nitrocellulose membrane (Protan BA85, 0.45µm, GE Healthcare) at 300mA for 2hrs. The membrane was blocked in PBS/5% Milk (Skim milk powder, LP0031, Oxiod) and incubated with primary antibodies for 1hr diluted in PBS/5% Milk, washed 3x 10min in PBS/0.1% Tween (P1379, Sigma-Aldrich) and incubated with the secondary antibody for 45min diluted in PBS/5% Milk and washed 3x in PBS/0.1% Tween. The blot was imaged by the Odyssey Classic imager (Li-Cor).

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Supplemental Information
Chapter 5

Table 1 | **List of E2s silenced in MelJuSo cells using a pool of four siRNA duplexes targeting each enzyme**

GeneID	LocusID	AccessionID
ATG3/APG3	64422	NM_022488
BIRC6	57448	NM_016252
UBE2A	7319	NM_003336
UBE2B	7320	NM_003337
UBE2C	11065	NM_007019
UBE2D1	7321	NM_003338
UBE2D2	7322	NM_003339
UBE2D3	7323	NM_003340
UBE2E1	7324	NM_003341
UBE2E2	7325	NM_152653
UBE2E3	10477	NM_006357
UBE2F/NCE2	140739	NM_080678
UBE2G1	7326	NM_003342
UBE2G2	7327	NM_003343
UBE2H	7328	NM_003344
UBE2I	7329	NM_003345
UBE2J1	51465	NM_016021
UBE2J2	118424	NM_058167
UBE2K/HIP2	3093	NM_005339
UBE2L3	7332	NM_003347
UBE2L6	9246	NM_004223
UBE2M	9040	NM_003969
UBE2N	7334	NM_003348
UBE2NL	389898	XM_372257
UBE2O/E2-230K	63893	NM_022066
UBE2Q_	55585	NM_017582
UBE2Q2/LOC92912	92912	NM_173469
UBE2R1/CDC34	997	NM_004359
UBE2R2	54926	NM_017811
UBE2S	27338	NM_014501
UBE2T/HSPC150	29089	NM_014176
UBE2U/MGC35130	148581	NM_152489
UBE2V1	7335	NM_021988
UBE2V2	7336	NM_003350
UBE2W/FLJ11011	55284	NM_018299
UBE2Z/FLJ13855	65264	NM_023079
UFC1	51506	NM_016406

Table 2 | **List of RNF26 E2 enzymes candidates**

GeneID	Function	Cellular localization	Domains *	Interacting E3s **
ATG3	Autophagy	Cytosol	-	-
UBE2G2	ERAD	ER/Cytosol	UBC	AMFR, RNF5, UBE3A
UBE2I	Mitosis	Nucleus/Cytosol	UBC	PIAS1, RANBP2
UBE2J1	ERAD	ER	UBC, 1xTM	SYVN1
UBE2L6	-	Cytosol	UBC	TRIM25, ARIH1
UBE2N	DNA repair	Nucleus/Cytosol	UBC	TRAF6, RNF8, STUB1
UBE2R1/CDC34	Mitosis	Nucleus/Cytosol	UBC	SKP2, RBX1, BTRC, CUL1, RNF11
UBE2V1	-	Nucleus/Cytosol	UBC	TRAF6, STUB1
UBE2Z	Apoptosis	Nucleus/Cytosol	UBC	UBR1, UBR2, UBR3
UFC1	Ufmylation	-	-	-
RNF26	LE transport	ER	RING, 4xTM	

* SMART prediction
** STRING database
UBC = Ubiquitin conjugating domain
TM = Transmembrane domain
U-box = Modified RING finger domain