# Antibody Characterization Report for
# E3 ubiquitin-protein ligase Itchy homolog (Itch)

**YCharOS Antibody Characterization Report**

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**Target:**

**Recommended protein name:** E3 ubiquitin-protein ligase Itchy homolog

**Recommended short name:** Itch

**Alternative protein name:** Atrophin-1-interacting protein 4, AIP4, HECT-type E3 ubiquitin transferase Itchy homolog, NFE2-associated polypeptide 1, NAPP1

**Gene name:** ITCH

**Uniprot:** Q96J02

We are a third-party organization with the mission to characterize commercial antibodies for all human protein through open science [1]. This report guides researchers to select the most appropriate antibodies for Itch. We used an antibody characterization pipeline [2] based on knockout (KO) cells to perform head-to-head comparisons of commercial antibodies for Itch by immunoblot (Western blot), immunoprecipitation and immunofluorescence. HeLa was selected based on evidence of appropriate Itch protein expression determined through public proteomics databases, namely PaxDB [3] and DepMap [4]. HeLa was modified with CRISPR/Cas9 to knockout the corresponding *ITCH* gene.

The authors do not provide an assessment of the quality of the tested antibodies as their respective performances are limited to our finite experimental conditions. The readers should interpret the present findings based on their own scientific expertise. The authors acknowledge that an antibody that demonstrates specificity in the stated test conditions can be suboptimal in a different experimental format or in cell lines that differ from those directly tested here.

**Table 1: Summary of the Itch antibodies tested**

| Company | Catalog number | Lot number | RRID (Antibody Registry) | Clonality | Clone ID | Host | Concentration (µg/µl) | Vendors recommended applications |
|---|---|---|---|---|---|---|---|---|
| Proteintech | 20920-1-AP | 49928 | AB_2878765 | polyclonal | - | rabbit | 0.23 | Wb,IP |
| Cell Signaling Technology | 12117** | 2 | AB_2797822 | recombinant-mono | D8Q6D | rabbit | 0.28 | Wb,IP |
| Developmental Studies Hybridoma Bank | CPTC-ITCH-1* | 2017-04-13 | AB_2617281 | monoclonal | CPTC-ITCH-1 | mouse | 0.027 | Wb |
| Thermo Fisher Scientific | PA5-65539 | WF3302109A | AB_2664412 | polyclonal | - | rabbit | 0.20 | Wb,IF |
| Thermo Fisher Scientific | PA5-18662 | WF3301701 | AB_10987202 | polyclonal | - | goat | 0.50 | Wb,IF |
| GeneTex | GTX02845** | 822104511 | AB_2915928 | recombinant-mono | GT1248 | rabbit | 0.80 | Wb |
| Aviva Systems Biology | ARP87308 | QC64641-42935 | AB_2915929 | polyclonal | - | rabbit | 0.50 | Wb |
| Abcam | ab280211** | GR3380046-1 | AB_2915930 | recombinant-mono | 32/ITCH | mouse | 1.11 | Wb |
| Abcam | ab108515** | GR42286-9 | AB_10860188 | recombinant-mono | EPR4936 | rabbit | 1.10 | Wb |

Wb=Western blot, IP= immunoprecipitation, IF=immunofluorescence, *=monoclonal antibody, **=recombinant antibody

**Table 2: Summary of the cell lines used**

| Institution | Catalog number | RRID (Cellosaurus) | Cell line | genotype |
|---|---|---|---|---|
| Montreal Neurological Institute | CCL-2 | CVCL_0030 | HeLa | WT |
| Montreal Neurological Institute | - | CVCL_B7MV | HeLa | *ITCH* KO |

**Figure 1: Itch antibody screening by immunoblot.**

Lysates of HeLa WT and *ITCH* KO were prepared, and 100 µg of protein were processed for immunoblot with the indicated Itch antibodies. The Ponceau stained transfers of each blot are shown. Antibody dilution used: 20920-1 at 1/1000, 12117** at 1/1000, CPTC-ITCH-1* at 1/90, PA5-65539 at 1/1000, PA5-18662 at 1/1000, GTX02845** at 1/1000, ARP87308 at 1/1000, ab280211** at 1/1000, ab108515** at 1/1000. Predicted band size: 102 kDa. *=monoclonal antibody, **=recombinant antibody

**Figure 2: Itch antibody screening by immunoprecipitation.**

HeLa lysates were prepared, and immunoprecipitation was performed using 2.0 µg of the indicated Itch antibodies pre-coupled to Dynabeads protein G or protein A. Samples were washed and processed for immunoblot with the indicated Itch antibody. For immunoblot, ab108515** and GTX02845** were used at 1/1000. The Ponceau stained transfers of each blot are shown. SM=10% starting material; UB=10% unbound fraction; IP=immunoprecipitate. *=monoclonal antibody, **=recombinant antibody

**Figure 3: Itch antibody screening by immunofluorescence.**

HeLa WT and *ITCH* KO cells were labelled with a green or a far-red fluorescent dye, respectively. WT and KO cells were mixed and plated to a 1:1 ratio on coverslips. Cells were stained with the indicated Itch antibodies and with the corresponding Alexa-fluor 555 coupled secondary antibody including DAPI. Acquisition of the blue (nucleus-DAPI), green (identification of WT cells), red (antibody staining) and far-red (identification of KO cells) channels was performed. Representative images of the merged blue and red (grayscale) channels are shown. WT and KO cells are outlined with yellow and magenta dashed line, respectively. Antibody dilution used: 20920-1 at 1/200, PA5-65539 at 1/200, PA5-18662 at 1/500, GTX02845** at 1/800, ARP87308 at 1/500, ab280211** at 1/1000, ab108515** at 1/1000. Bars = 10 µm. *=monoclonal antibody, **=recombinant antibody

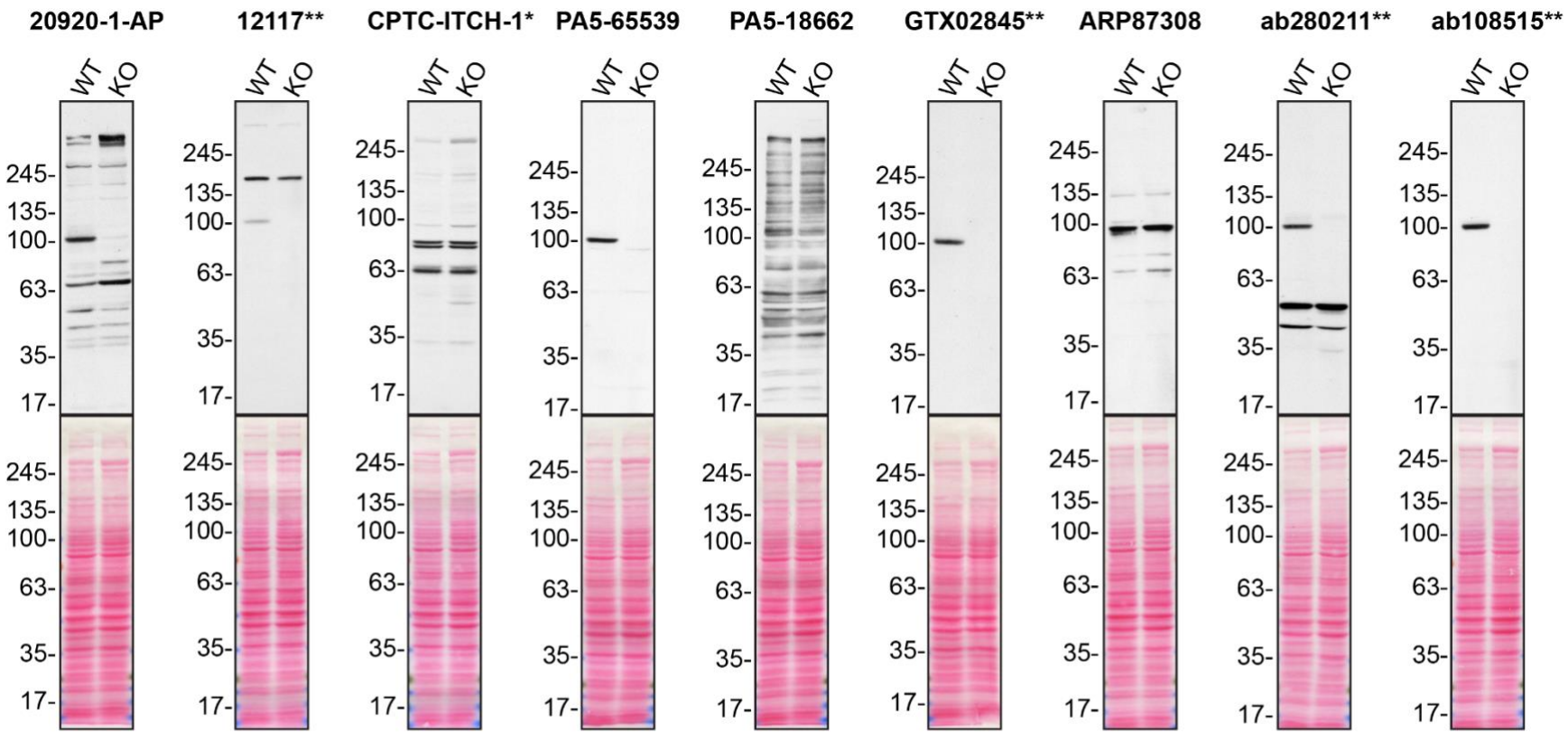


**Figure 1: Itch antibody screening by immunoblot**

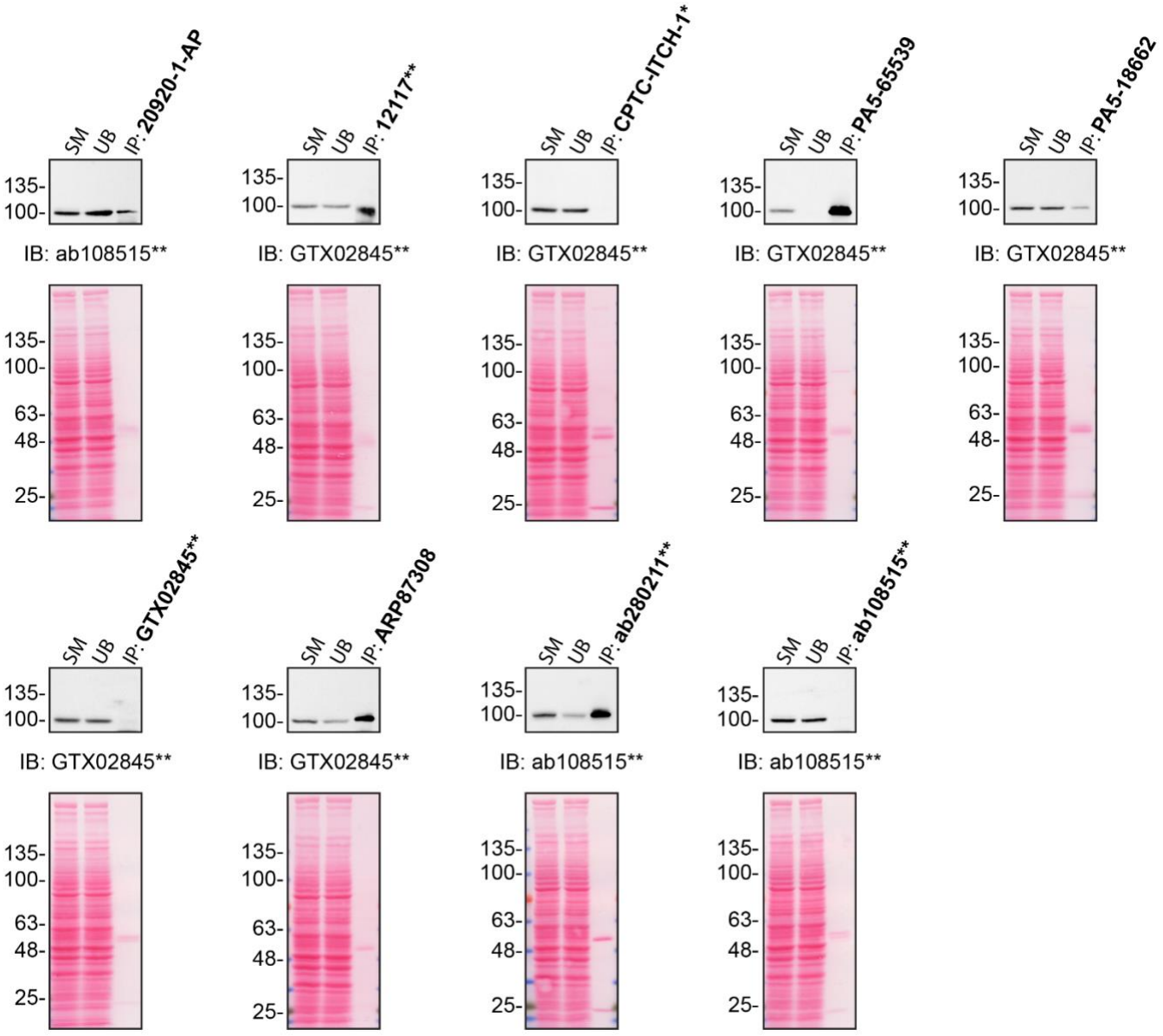


**Figure 2: Itch antibody screening by immunoprecipitation**

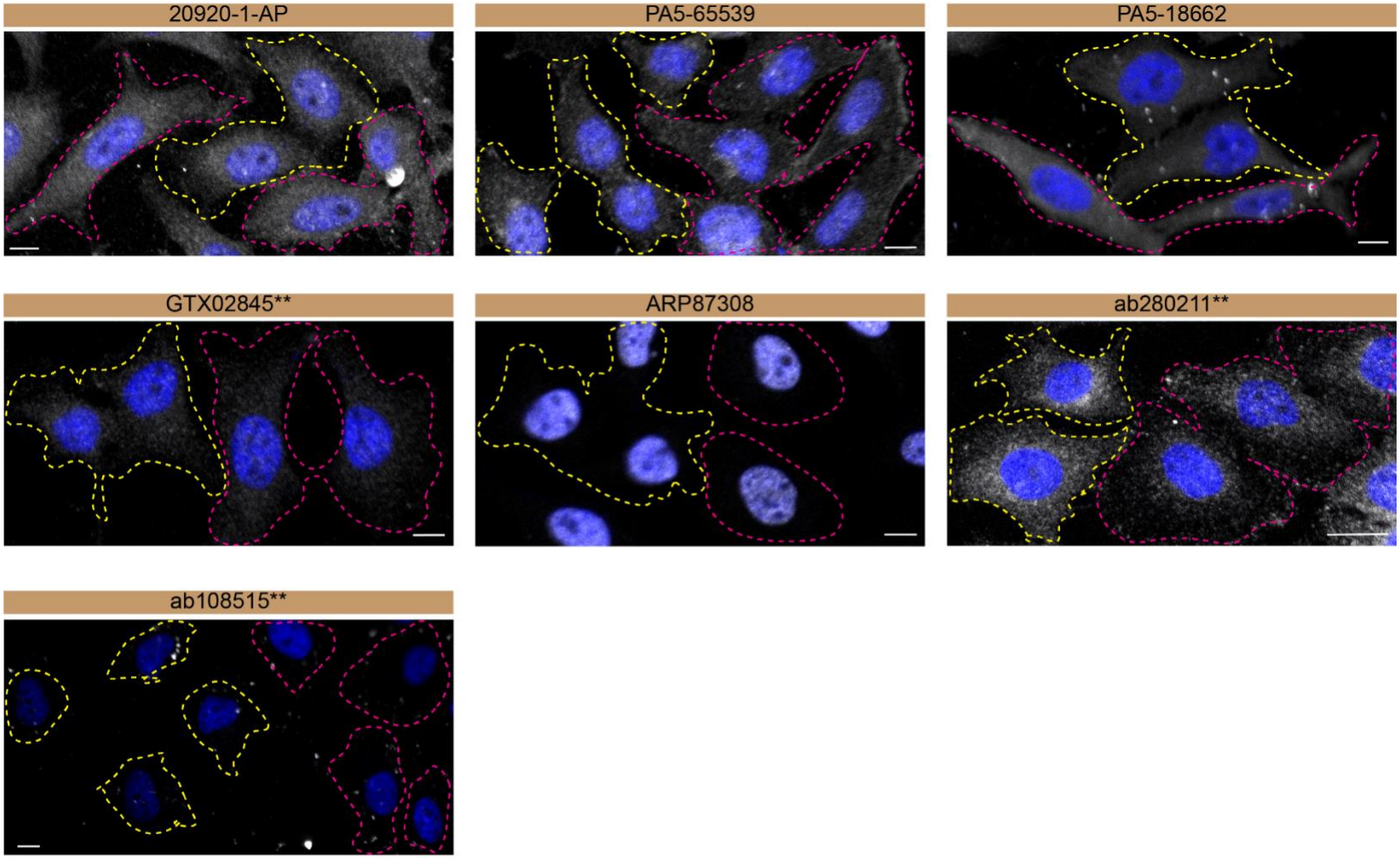


**Figure 3 : Itch antibody screening by immunofluorescence**

## Materials and methods

### Antibodies

All Itch antibodies are listed in Table 1. Peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies are from Thermo Fisher Scientific (cat. number 62-6520 and 65-6120). Alexa-555-conjugated goat anti-mouse and anti-rabbit secondary antibodies are from Thermo Fisher Scientific (cat. number A21424 and A21429).

### CRISPR/Cas9 genome editing

Cell lines used are listed in Table 2. HeLa *ITCH* KO clone was generated with low passage cells using the genome engineering CRISPR/Cas9 protocol [5]. Two guide RNAs were used to introduce a double-stranded break in the *ITCH* gene (sequence guide 1: GTGAAAATAGGAGAGTCAGT, sequence guide 2: TGAGAGCTGGAATACAAACC).

### Cell culture

Cells were cultured in DMEM high glucose (GE Healthcare cat. number SH30081.01) containing 10% fetal bovine serum (Wisent, cat. number 080450), 2 mM L-glutamate (Wisent cat. number 609065, 100 IU penicillin and 100 µg/ml streptomycin (Wisent cat. number 450201).

### Antibody screening by immunoblot

Immunoblots were performed as described in our standard operating procedure [6]. HeLa WT and *ITCH* KO were collected in RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor. Lysates were sonicated briefly and incubated 30 min on ice. Lysates were spun at ~110,000xg for 15 min at 4°C and equal protein aliquots of the supernatants were analyzed by SDS-PAGE and immunoblot. BLUelf prestained protein ladder from GeneDireX (cat. number PM008-0500) was used.

Immunoblots were performed with large 4-15% gradient polyacrylamide gels and transferred on nitrocellulose membranes. Proteins on the blots were visualized with Ponceau staining which is scanned to show together with individual immunoblot. Blots were blocked with 5% milk for 1 hr, and antibodies were incubated O/N at 4°C with 5% bovine serum albumin in TBS with 0,1% Tween 20 (TBST). Following three washes with TBST, the peroxidase conjugated secondary antibody was incubated at a dilution of ~0.2 µg/ml in TBST with 5% milk for 1 hr at room temperature followed by three washes with TBST. Membranes are incubated with ECL from

Pierce (cat. number 32106) prior to detection with HyBlot CL autoradiography films from Denville (cat. number 1159T41).

**Antibody screening by immunoprecipitation**

Immunoprecipitation was performed as described in our standard operating procedure [7]. Antibody-bead conjugates were prepared by adding 2 µg to 500 ul of Pierce IP Lysis Buffer from Thermo Fisher Scientific (cat. number 87788) in a microcentrifuge tube, together with 30µl of Dynabeads protein A- (for rabbit antibodies) or protein G- (for mouse antibodies) from Thermo Fisher Scientific (cat. number 10002D and 10004D, respectively). Pierce IP Lysis Buffer was supplemented with the Halt Protease Inhibitor Cocktail 100X from Thermo Fisher Scientific (cat. number 78446) at a final concentration of 1x. Tubes were rocked for ~2 hrs at 4°C followed by several washes to remove unbound antibodies.

HeLa WT were collected in Pierce IP buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol) supplemented with protease inhibitor. Lysates are rocked 30 min at 4°C and spun at 110,000xg for 15 min at 4°C. One ml aliquots at 0.75 mg/ml of lysate were incubated with an antibody-bead conjugate for ~2 hrs at 4°C. Following centrifugation, the unbound fractions were collected, and beads were subsequently washed three times with 1.0 ml of IP lysis buffer and processed for SDS-PAGE and immunoblot on 4-15% polyacrylamide gels.

**Antibody screening by immunofluorescence**

Immunofluorescence was performed as described in our standard operating procedure [8]. HeLa WT and *ITCH* KO were labelled with a green and a far-red fluorescence dye, respectively. The fluorescent dyes used are from Thermo Fisher Scientific (cat. number C2925 and C34565). WT and KO cells were plated on glass coverslips as a mosaic and incubated for 24 hrs in a cell culture incubator. Cells were fixed in 4% PFA (in PBS) for 15 min at room temperature and then washed 3 times with PBS. Cells were permeabilized in PBS with 0,1% Triton X-100 for 10 min at room temperature and blocked with PBS with 5% BSA, 5% goat serum and 0.01% Triton X-100 for 30 min at room temperature. Cells were incubated with IF buffer (PBS, 5% BSA, 0,01% Triton X-100) containing the primary Itch antibodies O/N at 4°C. Cells were then washed 3 × 10 min with IF buffer and incubated with corresponding Alexa Fluor 555-conjugated secondary antibodies in IF buffer at a dilution of 1.0 µg/ml for 1 hr at room temperature with DAPI. Cells were washed 3 × 10 min with IF buffer and once with PBS. Coverslips were mounted on a microscopic slide using fluorescence mounting media (DAKO).

Imaging was performed using a Zeiss LSM 700 laser scanning confocal microscope equipped with a Plan-Apo 40x air objective. Analysis was done using the Zen navigation software (Zeiss). All cell images represent a single focal plane. Figures were assembled with Adobe Illustrator.

## References


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