SUPPLEMENTARY INFORMATION

Supplementary methods

Materials

HEK 293T, and MEF derived cell lines were cultured in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin (GIBCO BRL). Antibodies were supplied by Abcam Limited (anti-VSV-G polyclonal antibodies), Sigma (anti VSV-CY3), BD Transduction Laboratories (anti-AIP4), Sigma (anti-Flag M2 and anti-Pan cadherin), Molecular Probes, Inc. (Alexa Fluor® conjugates) Amersham Biosciences (anti-mouse/anti-rabbit CY3) or provided by J.T. August (anti-LAMP1, obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242), H. Stenmark (Institute for Cancer Research, Oslo, Norway for the anti-Hrs). DTX-myc, AIP4 and AIP4DN (C830A) expression vectors were respective gifts of S. Artavanis-Tsakonas (Harvard Medical School, USA) and A. Atfi (INSERM U482, Paris, France). Cycloheximide, MG132 and Leupeptin were from Sigma.

Constructs

VSV tagged DTX and C3A DTX expression vectors : human DTX cDNA (a gift from S. Artavanis-Tsakonas) was amplified by PCR and inserted into GVSV-pCDNA3 (Brou *et al.*, 2000) at the NcoI and NotI sites. C3A (where the Cysteines 411, 468 and 471 are replaced by Alanines) was obtained by sequential site directed mutagenesis. DTX-VSV was cloned by PCR into the retroviral expression vector MoCAG (a gift from P. Charneau, Pasteur Institute) under the control of the CAG promoter.

Ubiquitin expression vectors : WT, or mutated ubiquitin cDNAs (K29, K48 or K63, generous gifts from R. Baer, Columbia University, New York) were amplified by PCR and inserted into GVSV-pCDNA3 at the EcoR1 and Xba1 sites.

siAIP4 vector: a BamH1-Xho1 fragment of pSUPER vector, containing the H1 RNA promoter, was cloned into pCDNA3 at the Bgl2 and Xho1 sites to replace the CMV promoter, then an oligonucleotide was inserted at the Bgl2 and Hind3 sites. This vector allows synthesis of a hairpin RNA, which is matured in the cells to AIP4-specific siRNA, corresponding to nucleotides 190-211 relative to the start codon of human AIP4 cDNA , that was previously described (Marchese *et al.*, 2003). All constructs were checked by sequencing.

Cell lines establishment

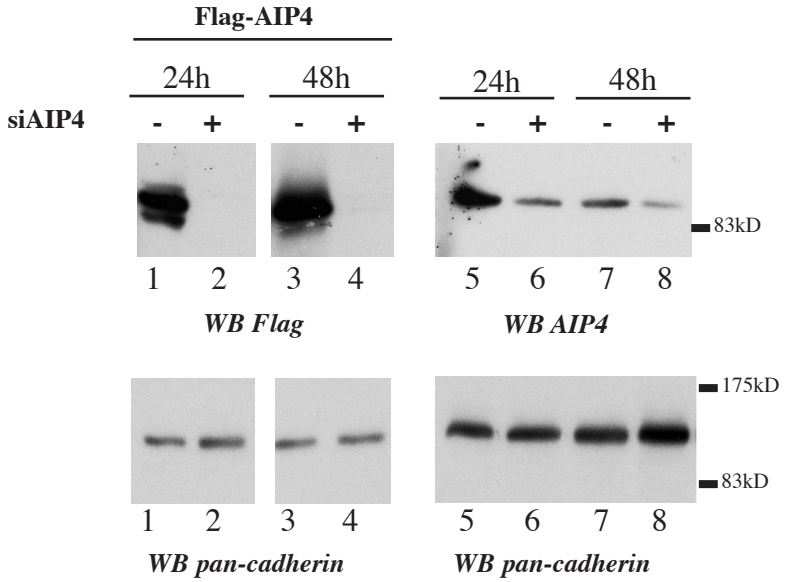
High titers of recombinant DTX-VSV-MoCAG viruses were obtained after transfection of the Plat-E ecotropic packaging cell line. Retroviruses-containing supernatant were collected 48h after transfection and added to MEF cells derived from WT or Itch-/- mice (named respectively M or I cell lines). The retroviral transduction protocol was repeated three times to obtain the MD and ID pools of cells.

Supplementary references

Brou C, *et al*. (2000) A novel proteolytic cleavage involved in Notch signaling: The role of the disintegrin-metalloprotease TACE. *Mol Cell* **5:** 207-216

Brummelkamp TR, Nijman SMB, Dirac AMG, Bernards R (2003) Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-[kappa]B. *Nature* **424:** 797-805

Supplementary figure S1

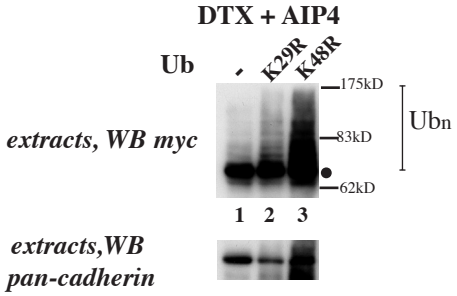


Supplementary figure S1 : Reduction of AIP4 level by siRNA silencing.

An AIP4-specific siRNA sequence (Marchese, 2003) was cloned into a pSuper-derived vector (Brummelkamp, 2003). 293T cells were transfected either with siAIP4-expressing plasmid (even numbered lanes) or with the control plasmid (odd numbered lanes) alone (lanes 5-8), or together with an AIP4 expression vector (lanes 1-4). Twenty four or 48h after transfection, the amount of exogenous or endogenous AIP4 was tested by western blotting of cell lysates. The top panels show the levels of AIP4 (overexpressed detected with anti-Flag antibody, endogenous detected with anti-AIP4 antibody), while the bottom panels show pan-cadherin as a loading and siRNA specificity control.

Transfection of the AIP4-specific siRNA vector effectively reduced the levels of either overexpressed (lanes 1 to 4) or endogenous AIP4 (lanes 5 to 8) as compared with the empty vector (lanes 1, 3, 5, 7).

Supplementary figure S2

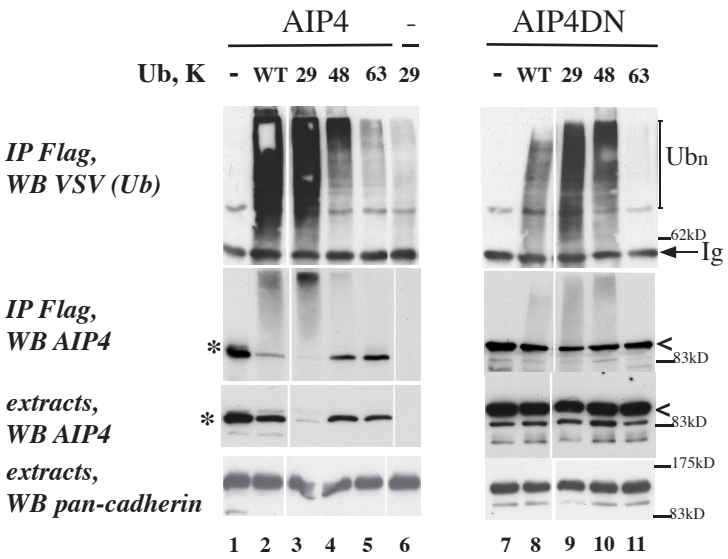


Supplementary figure S2 : DTX ubiquitination mostly depends on the presence of Lysine 29 of ubiquitin.

293T cells were transfected with AIP4 and myc-DTX (lane 1), together with plasmids allowing expression of Flag-tagged ubiquitin, either mutated in lysine 29 (indicated as K29R in lane 2), or in lysine 48 (indicated as K48R in lane 3). Extracts were analyzed by SDS-PAGE (6.5%) and successively immunoblotted with antibodies against myc and pan-cadherin as indicated on the left. Ubn indicates the polyubiquitin chains.

A ladder of polyubiquitinated DTX was detected with K48R ubiquitin (upper panel, lane 3), suggesting that in the presence of AIP4, most of the chains formed on DTX do not require linkage through lysine 48 of ubiquitin. On the other hand, mutation of K29 of ubiquitin led to a reduction in the quantity of ubiquitinated DTX (lane 2) to a level close to that observed in the absence of cotransfected Ub (lane 1). This result is in accordance with the main ubiquitination of DTX occurring via K29-linked chains.

Supplementary figure S3



Supplementary figure S3 : Ubiquitination of AIP4.
293T cells were transfected with Flag-tagged AIP4 (lanes 1-5), or AIP4DN (lanes 7-11), together with a plasmid allowing expression of VSV-tagged ubiquitin, either WT (lanes 2, 8), or mutated in all but lysine 29 (indicated as 29 in lanes 3, 6, 9), in all but lysine 48 (indicated as 48 in lanes 4 and 10) or in all but lysine 63 (indicated as 63 in lanes 5 and 11). Extracts or AIP4 immunoprecipitates (IP Flag) were analyzed by SDS-PAGE (6.5%) and successively immunoblotted with antibodies against VSV, AIP4 and pan-cadherin as indicated on the left. The arrow indicates the immunoglobulin heavy chain (Ig) and Ubn the polyubiquitin chains. White lines indicate that intervening lanes have been spliced out.

By immunoprecipitating AIP4 from transfected cells and immunoblotting for exogenous ubiquitin with anti-VSV antibody, we detected a strong signal with WT ubiquitin (upper panel, lane 2). This signal was in comparison very weak when using extracts derived from AIP4DN transfected cells (lane 8), although the amount of protein in the extract was higher (third row of panels from top), therefore suggesting that the observed smear in lane 2 was due to autoubiquitination of AIP4. Furthermore, we observed the same strong signal when using Ub K29 (lane 3), whereas polyubiquitination was much weaker when using Ub K48 or K63 (lanes 4, 5). The very different intensities of the signals obtained with Ub WT or K29 on one hand, and Ub K48 or K63 on the other hand, suggest that AIP4 essentially directs the formation of unconventional linkages involving residue K29 of ubiquitin. AIP4DN exhibited much less ubiquitination, the residual signal being possibly due to ubiquitination catalyzed by endogenous AIP4, or to the action of another E3 ligase. Reblotting of the immunoprecipitates or of the total extracts with anti-AIP4 antibody revealed that AIP4DN was uniformly present in all extracts (second and third row of panels). On the other hand, the presence of excess WT or K29 ubiquitin led to a decrease in the quantity of native AIP4 immunoprecipitated (second panel), probably because most molecules have been ubiquitinated and degraded. This increased degradation of AIP4 in the presence of overexpressed ubiquitin was detected, although less notably, by direct immunoblotting of the extracts with anti-AIP4 antibody (third panel) and normalization with a non-related endogenous protein (pan-cadherin, fourth panel). This result is in accordance with the hypothesis of overexpressed AIP4 targeting its own degradation after autoubiquitination via K29-linked chains.