Isoform-specific monoubiquitination, endocytosis, and degradation of alternatively spliced ErbB4 isoforms

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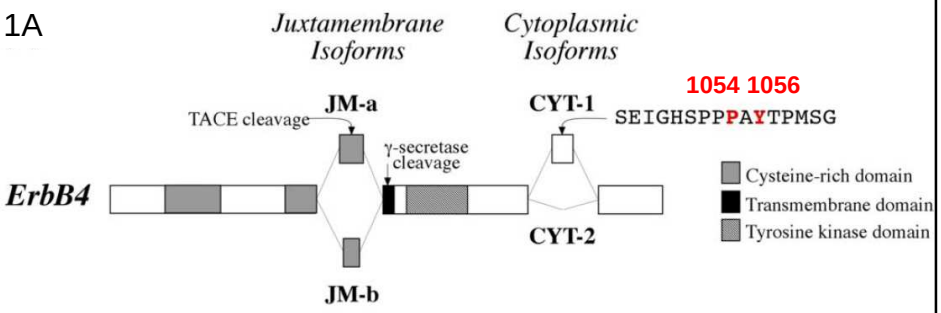
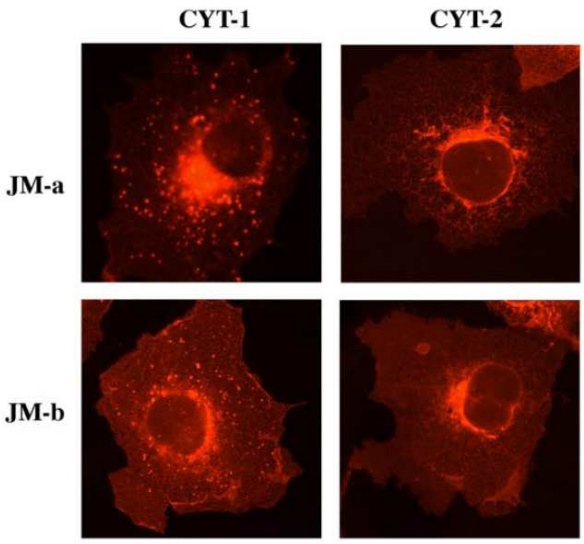
Results

● Constructs encoding all four full-length ErbB4 isoforms, i.e., the four possible combinations of alternative JM and CYT domains (Fig. 1A), with C-terminal HA tags were transiently expressed in COS-7 cells

1A



● COS-7 cells expressing each of the HA-tagged ErbB4 isoforms were stained with anti-HA antibody (red) and photographed under a fluorescence microscope.

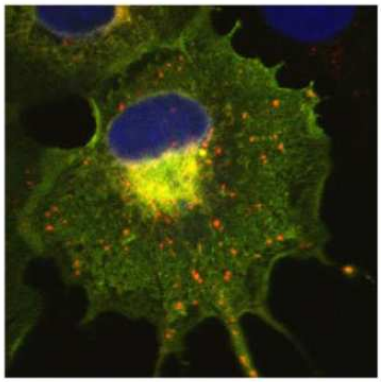


● Isoforms with the CYT-1 type were more frequently localized into cytoplasmic vesicles than CYT-2 isoforms, irrespective of the JM domain.

● Both the cleavable JM-a CYT-1 and the noncleavable JM-b CYT-1 localized to the vesicles. These data indicate the presence of the intact full-length ErbB4 within the vesicular structures.

● Similar results were obtained with ErbB4 constructs without tags or with C-terminal EGFP tags, with variable expression levels, and in the backgrounds of HeLa, NIH 3T3, and MCF-7 cells

● COS-7 cells simultaneously expressing **HA-tagged ErbB4 JM-a CYT-1** and **Myc-tagged ErbB4 JM-a CYT-2** were stained with anti-HA (red) and anti-Myc (green) antibodies. The nuclei were stained with **DAPI** (blue)

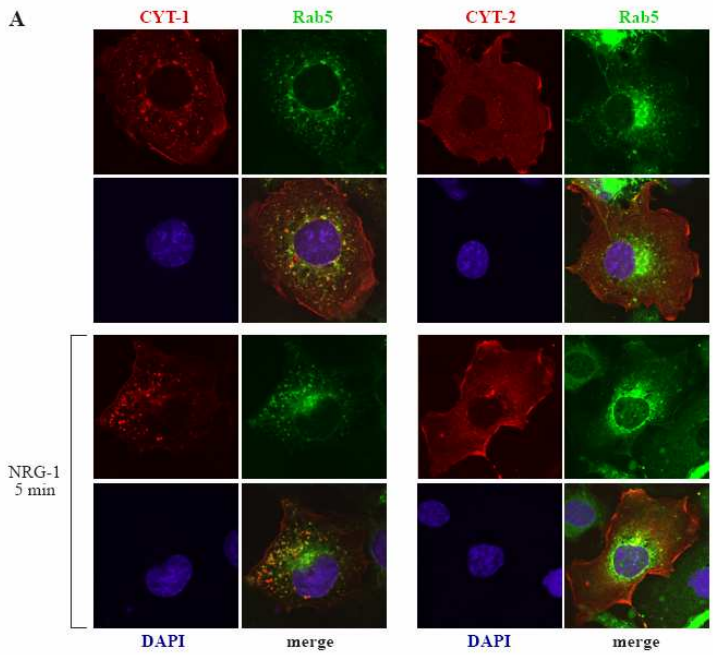


CYT-1
CYT-2
DAPI

● Differential subcellular localization of ErbB4 CYT isoforms was evident in confocal analysis of single cells simultaneously expressing differentially tagged CYT-1 or CYT-2 isoforms.

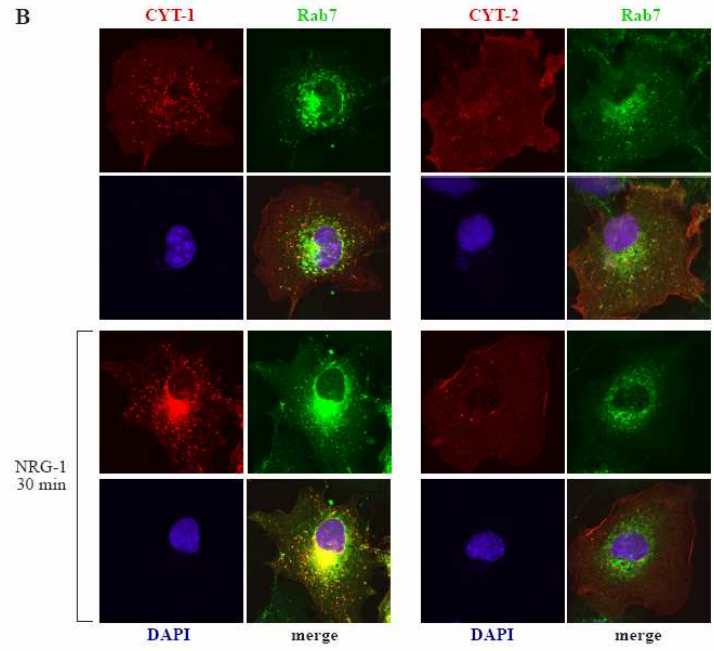
● To determine the identity of CYT-1-positive vesicles, **HA-tagged ErbB4 CYT-1** or **CYT-2** was expressed in COS-7 cells together with **GFP-tagged Rab5** or **Rab7**, markers of early and late endosomal vesicles, respectively.

Rab5
Early endosomal vesicle marker



Confocal microscopy demonstrated that ErbB4 CYT-1 isoforms, but not CYT-2 isoforms, clearly colocalized with Rab5 in intracellular vesicular structures

Rab7
Late endosomal vesicle marker

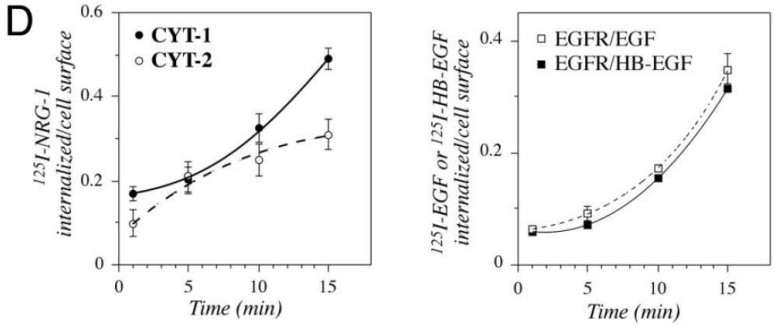


Confocal microscopy demonstrated that ErbB4 CYT-1 isoforms, but not CYT-2 isoforms, clearly colocalized with Rab7 in intracellular vesicular structures

ErbB4 CYT-1 is internalized and degraded more efficiently than ErbB4 CYT-2.

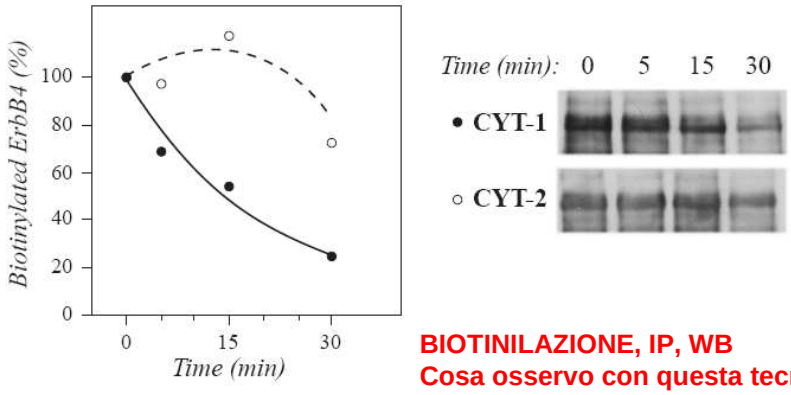
● To address whether the more efficient localization into endocytic vesicles was associated with enhanced receptor internalization, the uptake of ^{125}I-NRG-1 was measured by using NIH 3T3-7d transfectants stably expressing the noncleavable ErbB4 JM-b CYT-1 or JM-b CYT-2.

● Internalization of ^{125}I-NRG-1 was significantly faster in cells expressing CYT-1 than in cells expressing CYT-2 (Left). The kinetics of ligand-stimulated internalization by CYT-1 was similar to that of EGFR known to undergo efficient internalization after ligand stimulation (Right).



Cells were incubated for 1, 5, 10, or 15 min with 125I-NRG-1, 125I -EGF, or 125I-HB-EGF at 37℃. Cells were washed once with PBS and twice with an acidic buffer and lysed with NaOH. Radioactivity removed in acid washes containing surface-bound growth factors was compared with radioactivity in cell lysates containing internalized growth factors.

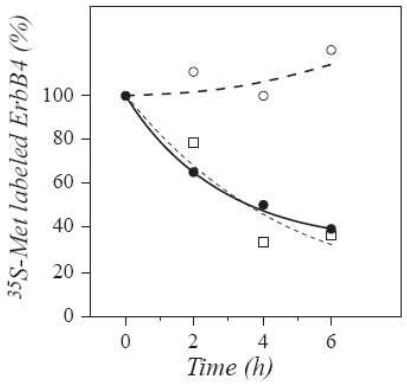
● Consistent with differential internalization, NRG-1 stimulation induced faster down-regulation of biotinylated cell surface-associated pool of ErbB4 CYT-1 when compared with ErbB4 CYT-2.

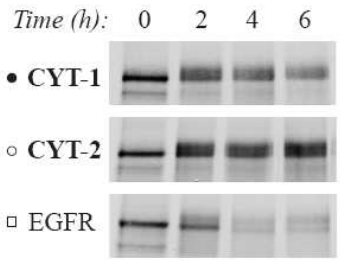


BIOTINILAZIONE, IP, WB
Cosa osservo con questa tecnica?

COS-7 cells were tranfected with constructs encoding ErbB4 JM-a CYT-1 or ErbB4 JM-a CYT-2. After 6 h starvation without FCS, cells were stimulated with 50 ng/ml NRG-1 for 0, 5, 15, or 30 min, and incubated on ice in the presence of biotin for 45 min.
Biotinylated cell surface-associated ErbB4 was detected by immunoprecipitation with anti-ErbB4 antibody (HFR-1) and Vectastain ABC kit.

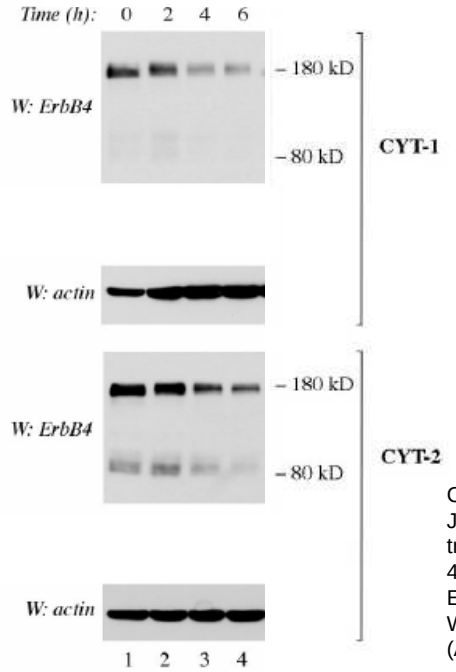
● To address the functional outcome of differential internalization, degradation of ^{35}S-methionine-labeled noncleavable **ErbB4 JM-b CYT-1** and **JM-b CYT-2** was determined in COS-7 cells.
● Degradation of CYT-1 was significantly more effective than degradation of CYT-2. Again, the basal degradation rate of ErbB4 CYT-1, but not of ErbB4 CYT-2, was similar to that of EGFR.





INCORPORAZIONE di ^{35}S, IP, WB
Cosa osservo con questa tecnica?

COS-7 cells were tranfected with constructs encoding ErbB4 JM-b CYT-1, ErbB4 JM-b CYT-2, or EGFR. Cells were labeled with ^{35}S-methionine, washed and lysed 0, 2, 4, and 6 h after labeling. Lysates were immunoprecipitated with anti-EGFR (Santa Cruz Biotechnology) or anti-ErbB4 antibodies (HFR-1) and analyzed by autoradiography of SDS/PAGE gels.



● CYT-1 was degraded more efficiently than CYT-2 also when stability of cleavable JM-a isoforms was analyzed by blocking protein synthesis with cycloheximide.

● These data indicate that CYT-1 isoforms are more efficiently internalized than CYT-2 isoforms and that the difference in internalization is associated with a difference in receptor degradation rate.

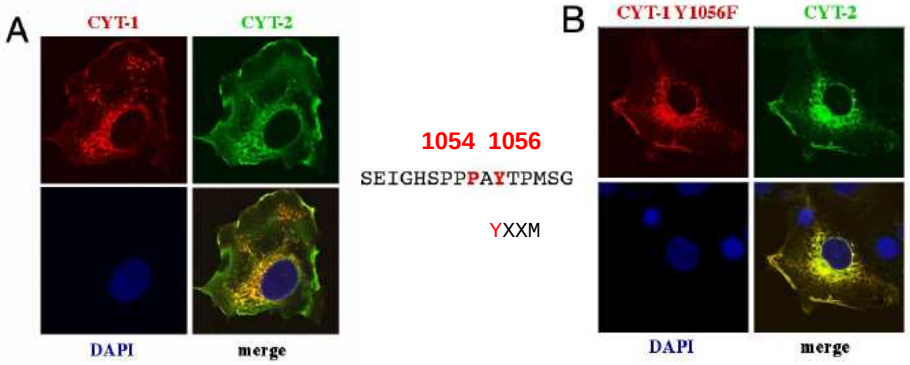
COS-7 cells expressing cleavable ErbB4 JM-a CYT-1 or ErbB4 JM-a CYT-2 were treated with 100 µM cycloheximide for 0, 2, 4, or 6 h and lysed.
ErbB4 expression was analyzed by Western blotting with anti-ErbB4 antibody (Abcam).

PPXY motif within the CYT-1-specific sequence is necessary for endocytosis of ErbB4 CYT-1

● CYT-1 isoforms differ from CYT-2 isoforms structurally by containing an insert including one tyrosine residue, Y1056, that functions as the only direct docking site of ErbB4 for PI3-K.

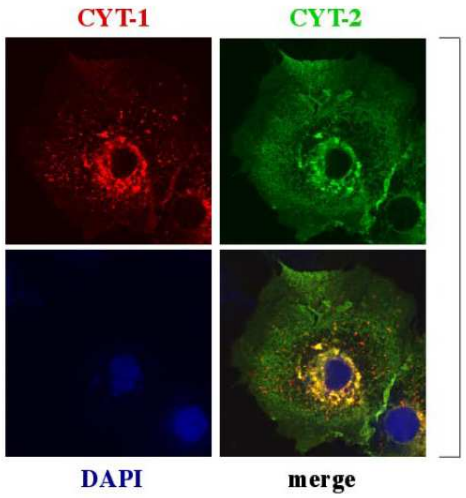
● The Y1056 residue is localized within a **YXXM** consensus binding motif for PI3-K that spans the amino acids 1056–1059 as **YTPM** sequence in CYT-1. Modification of phosphatidyl inositols by PI3-K may regulate endocytosis.

● When CYT-1 Y1056F was expressed simultaneously with wild-type CYT-2, the difference in the intracellular targeting of the CYT isoforms was abolished.



COS-7 cells were transfected with constructs encoding wild-type **Myc-tagged ErbB4 JM-a CYT-2** and **wild-type (**or **Y1056F) HA-tagged ErbB4 JM-a CYT-1**.

● However, inhibition of PI3-K activity with the chemical inhibitor LY294002 did not prevent the localization of CYT-1 into the cytoplasmic vesicles whereas it efficiently blocked NRG-1-stimulated phosphorylation of Akt (data not shown).

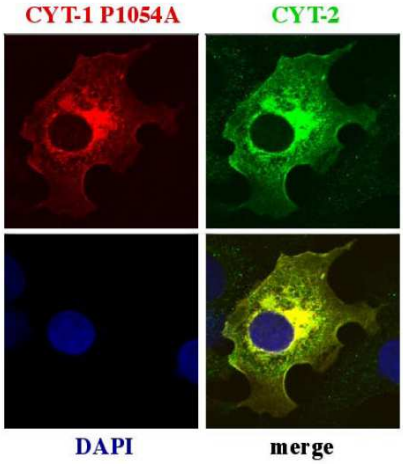


+LY294002

Cells were treated for 4 h with 20 μM LY294002 to inhibit PI3-K activity.

● These data demonstrate that the Y1056 residue, but not coupling of PI3-K activity to Y1056, was involved in regulating differential targeting of the CYT isoforms.

● The CYT-1-specific stretch of 16 aa also includes a PPXY motif that may in principle serve as a binding site for WW domain containing proteins. The PPXY motif is located between amino acids 1053 and 1056 as a PPAY sequence in CYT-1 and thus shares the Y1056 with the YTPM binding sequence for PI3-K.
● To assess whether the results obtained with the Y1056F mutation were a consequence of disrupting a functional PPXY motif, rather than a YXXM motif for PI3-K, the proline-1054 was mutated to alanine in the CYT-1-specific sequence.
Indeed, P1054A mutation abolished the appearance of CYT-1-positive vesicles.



1054 1056

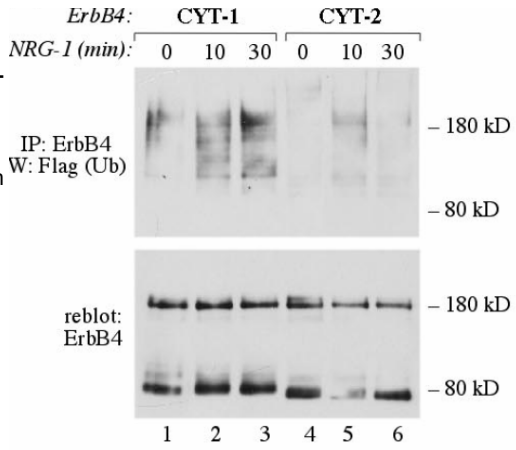
SEIGHSPPPAYTPMSG

● These data suggest that the PPXY motif present in ErbB4 CYT-1 isoforms functions as an endocytic sorting motif.

PPXY motif within the CYT-1-specific sequence is necessary for efficient ubiquitination of ErbB4 CYT-1.

Ubiquitination is one of the key regulators of endocytosis and subsequent lysosomal degradation of cell surface receptors in both yeast and mammalian cells.

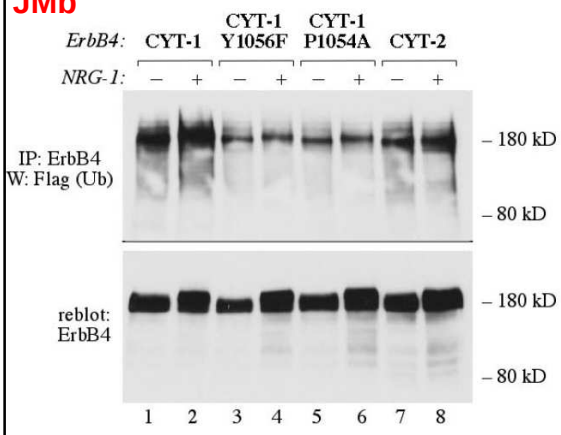
COS-7 cells expressing Myc-tagged ErbB4 **JM-a CYT-1** or ErbB4 **JM-a CYT-2** and **Flag-tagged ubiquitin** were stimulated for 0, 10, or 30 min with 50 ng/ml NRG-1.
Lysates were immunoprecipitated with anti-ErbB4 antibody (HFR-1) followed by Western blotting with anti-Flag antibody. Membrane was reblotted with anti-ErbB4 antibody (sc-283).



● full length JM-a CYT-1 was more efficiently ubiquitinated than full length JM-a CYT-2.

The CYT-1-specific sequence of 16 aa does not contain lysine residues, ruling out a difference in availability of direct ubiquitination sites. To address the significance of the CYT-1-specific PPXY motif, ubiquitination of the CYT-1 Y1056F and P1054A mutants was tested. Consistent with the effect on targeting to cytoplasmic vesicles, both mutants were ubiquitinated to a lesser extent when compared with wild-type CYT-1.

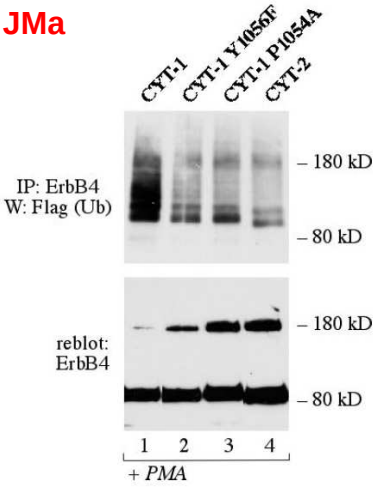
JMb



COS-7 cells expressing HA-tagged ErbB4 were stimulated for 0 or 30 min with 50 ng/ml NRG-1. Lysates were immunoprecipitated with anti-ErbB4 antibody (HFR-1) followed by Western blotting with anti-Flag antibody. Membrane was reblotted with anti-ErbB4 antibody (Abcam).

This was observed both in the context of full-length non cleavable JM-b isoforms and in the context of m80 fragments generated from full-length JM-a isoforms by PMA stimulation.

JMa



COS-7 cells expressing ErbB4 and Flag-tagged ubiquitin were stimulated for 0 or 30 min with 100 ng/ml PMA. Lysates were immunoprecipitated with anti-ErbB4 antibody (HFR-1) followed by Western blotting with anti-Flag antibody. Membrane was reblotted with anti-ErbB4 antibody (sc-283).

PPXY motif within the CYT-1-specific sequence serves as a binding site for WW domains of the E3 Ubiquitin ligase Itch

1- the membrane-anchored m80 fragments of CYT-1 and CYT-2 type are ubiquitinated to different extents

2- there is no difference between the isoforms when ubiquitination of the soluble ICDs (s80s) is analyzed

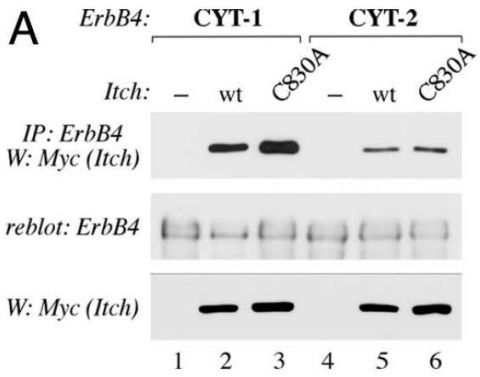
3- the efficient ubiquitination of membrane anchored CYT-1 isoforms depended on an intact PPXY motif

● Together these observations imply that the ubiquitin ligase responsible for CYT-1-specific modification contains a membrane anchor, as well as a WW domain capable of interacting with the PPXY motif.

● One family of ubiquitin ligases that contain both C2 domains for coupling to membrane phospholipids and two or more WW domains is the Nedd4 family of HECT-type E3 ubiquitin ligases.

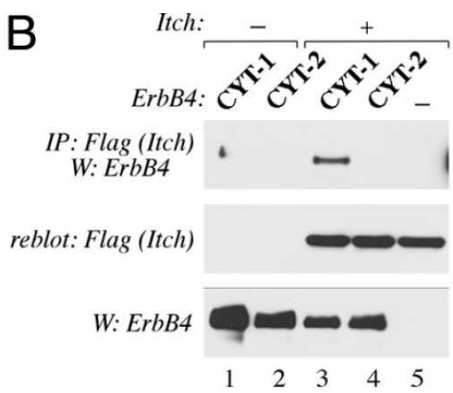
To test for an interaction between ErbB4 and two candidate members of the Nedd4 family, coimmunoprecipitation of ErbB4 together with either Nedd4 or Itch was analyzed.
Nedd4 failed to demonstrate association (data not shown),
Itch associated with ErbB4 in reciprocal coimmunoprecipitation experiments (3A and 3B).

Although some association was also observed between Itch and CYT-2 isoforms consistently more CYT-1 than CYT-2 coprecipitated with Itch.



COS-7 cells were transfected with constructs encoding ErbB4 **JM-a CYT-1** or ErbB4 **JM-a CYT-2** and Myc-tagged Itch or Itch C830A (catalitically inactive). Lysates were immunoprecipitated with anti-ErbB4 antibody (HFR-1) followed by Western blotting with anti-Myc antibody. Membrane was reblotted with anti-ErbB4 antibody (sc-283). Expression of Itch was analyzed by Western blotting with anti-Myc antibody.

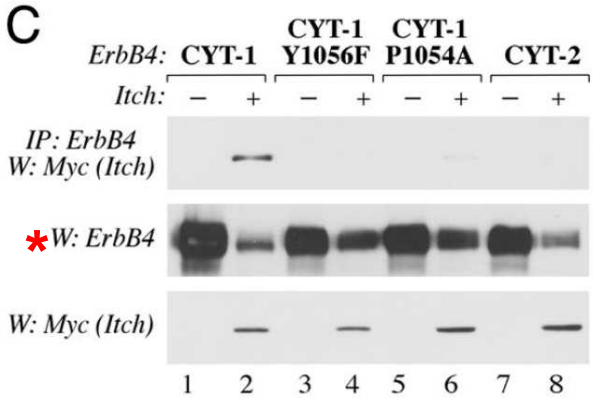
The interaction between Itch and either ErbB4 isoform was not dependent on the catalytic activity of the Itch HECT domain, because the catalytically inactive Itch mutant C830A demonstrated equal binding when compared with wild-type Itch



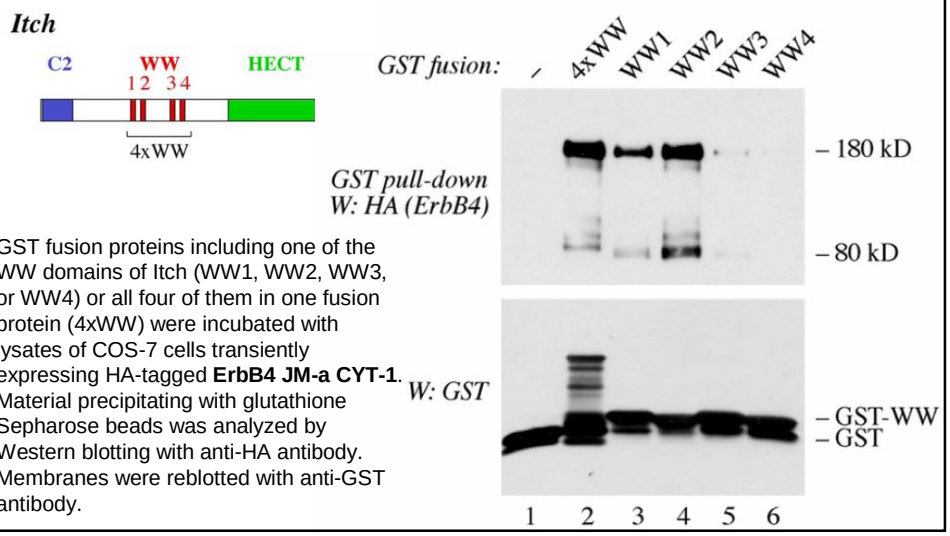
COS-7 cells were transfected with constructs encoding HA-tagged ErbB4 **JM-a CYT-1** or ErbB4 **JM-a CYT-2** and **Flag-tagged Itch**. Lysates were immunoprecipitated with anti-Flag antibody followed by Western blotting with anti-ErbB4 antibody (Abcam). Membrane was reblotted with anti-Flag antibody. Expression of ErbB4 was analyzed by Western blotting with anti-ErbB4 antibody (Abcam).

COS-7 cells were transfected with constructs encoding ErbB4 **JM-a CYT-1**, **JM-a CYT-1 Y1056F**, **JM-a CYT-1 P1054A**, or **JM-a CYT-2** with or without construct for Myc-tagged Itch.
Lysates were immunoprecipitated with anti-ErbB4 antibody (HFR-1) followed by Western blotting with anti-Myc antibody. Membrane was reblotted with anti-ErbB4 antibody (sc-283). Expression of Itch was analyzed by Western blotting with anti-Myc antibody.

The interaction between Itch and CYT-1 depended on an intact CYT-1-specific PPXY motif as mutating either Y1056 or P1054 attenuated binding.

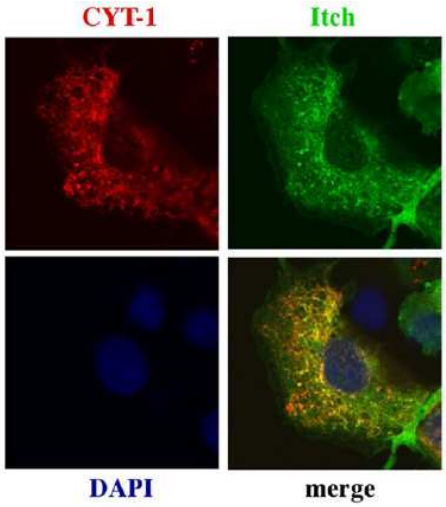


* Perché le bande di ErbB4 hanno diversa intensità?



GST fusion proteins including one of the WW domains of Itch (WW1, WW2, WW3, or WW4) or all four of them in one fusion protein (4xWW) were incubated with lysates of COS-7 cells transiently expressing HA-tagged **ErbB4 JM-a CYT-1**. Material precipitating with glutathione Sepharose beads was analyzed by Western blotting with anti-HA antibody. Membranes were reblotted with anti-GST antibody.

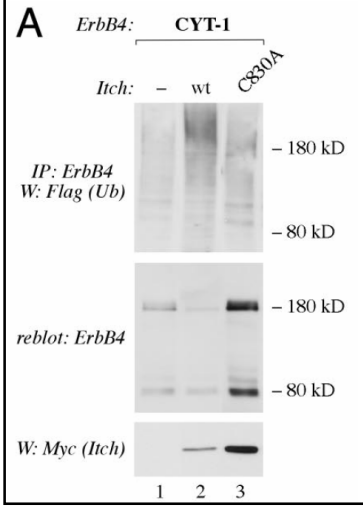
Confocal microscopy analysis also demonstrated colocalization of Itch with CYT-1 isoforms within cytoplasmic vesicular structures.



COS-7 cells simultaneously expressing HA-tagged ErbB4 JM-b CYT-1 and Myc-tagged Itch were stained with anti-HA (red) and anti-Myc (green) antibodies. The nuclei were stained with DAPI (blue).

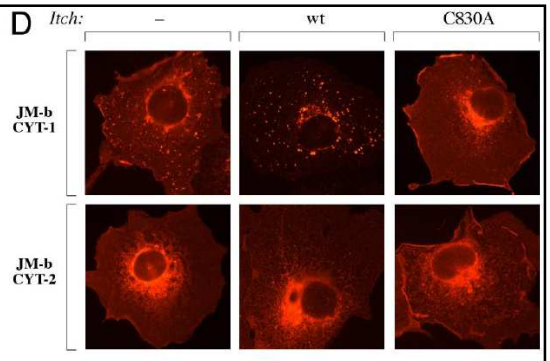
All together, these data show that ErbB4 interacts with the WW domain-containing E3 ubiquitin ligase Itch and that ErbB4 CYT-1 associates with Itch more avidly than CYT-2 because of an interaction via the CYT-1-specific PPXY motif.

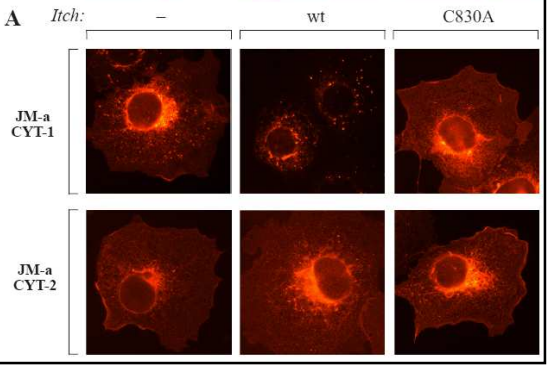
Itch monoubiquitinates ErbB4.
To analyze the functional significance of the interaction between ErbB4 and Itch, the role of Itch in ubiquitination of ErbB4 was analyzed. Expression of Itch increased ubiquitination of ErbB4 unlike the catalytically inactive C830A mutant of Itch (Fig. 4*A*), which was still capable of binding ErbB4 (Fig. 3*A*).
These observations suggest that Itch functions as an E3 ubiquitin ligase for ErbB4 and Itch C830A as a dominant negative inhibitor of ErbB4 ubiquitination.



COS-7 cells expressing ErbB4 **JM-a CYT-1** and **Flag-tagged ubiquitin** in the presence or absence of **Myc-tagged Itch** or **Itch C830A** were lysed, and the lysates were immunoprecipitated with anti-ErbB4 antibody (HFR-1) followed by Western blotting with anti-Flag antibody. Membrane was reblotted with anti-ErbB4 antibody (sc-283). Expression of Itch was analyzed by Western with anti-Myc antibody.

Overexpression of wildtype Itch enhanced the localization of CYT-1, but not CYT-2, isoforms into cytoplasmic vesicles regardless of the type of the JM domain.

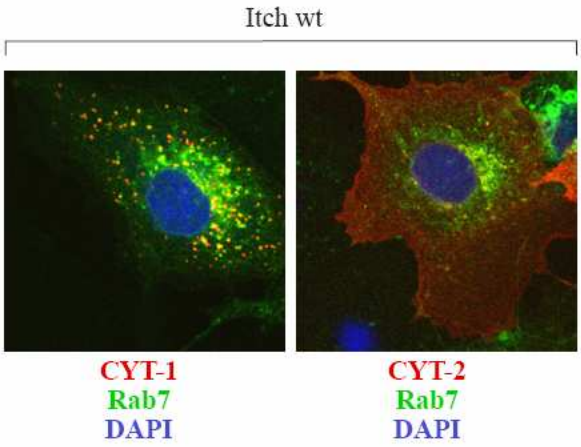




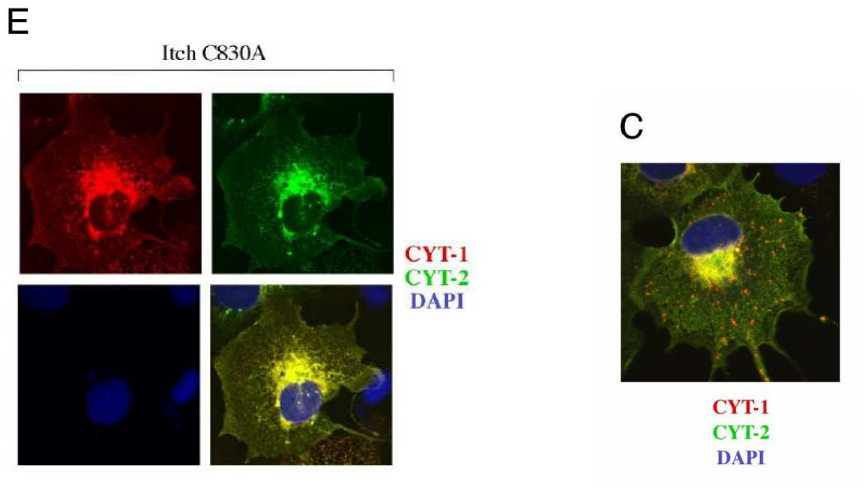
COS-7 cells expressing HA-tagged ErbB4 in the presence or absence of Myc-tagged Itch or Itch C830A were stained with anti-HA antibody (red) and photographed under a fluorescence microscope.
Itch expression was confirmed by staining with anti-Myc antibody (data not shown).

The specifically CYT-1-positive vesicles induced by Itch overexpression were for the most part also positive for Rab7 expression, supporting a role for Itch in targeting CYT-1 into late endosomal/lysosomal pathway.

COS-7 cells were transfected with constructs encoding **HA-tagged ErbB4 JM-b CYT-1** (*Left*) or **HA-tagged ErbB4 JM-b CYT-2** (*Right*) and **Myc-tagged Itch** and **GFP-Rab7**. ErbB4 (anti-HA antibody; red), Rab7 (GFP; green) and nuclei (DAPI; blue) were visualized with confocal microscopy.



- to more directly analyze the significance of Itch in the differential vesicular targeting of CYT-1 and CYT-2 isoforms, full-length CYT-1 and CYT-2 coupled to different epitope tags were simultaneously overexpressed together with the dominant negative Itch C830A.
- under these conditions neither CYT-1 nor CYT-2 localized to cytoplasmic vesicles (compare Fig. 4*E* with Fig. 1*C*).



SUMMARY

● endocytosis of ErbB4 is regulated in an isoform-specific manner:

- CYT-1 isoforms were efficiently endocytosed
- CYT-2 isoforms were endocytosis-impaired

● CYT-1 isoforms in endocytic vesicles colocalized with Rab5 and Rab7 indicating trafficking via early endosomes to late endosomal/lysosomal structures

● a PPXY motif within the CYT-1-specific sequence that lacks from CYT-2 is necessary both for ubiquitination and endocytosis of CYT-1 isoforms and provided a binding site for a WW domain-containing ubiquitin ligase Itch

● Itch catalyzed ubiquitination of ErbB4 CYT-1, promoted its localization into intracellular vesicles, and stimulated degradation of ErbB4 CYT-1

● Dominant negative Itch suppressed ErbB4 CYT-1 endocytosis and degradation.

● These data indicate that ErbB4 isoforms differ in endocytosis and degradation by a mechanism mediated by CYT-1-specific PPXY motif interacting with a WW domain-containing E3 ubiquitin ligase

CONCLUSIONS

● the ErbB4 isoforms are different in their susceptibility to internalization, monoubiquitination, endocytic targeting, and degradation

● The molecular mechanism of this variation is the differential association of a PPXY motif within the CYT-1 isoform-specific sequence with a Nedd4 family E3 ubiquitin ligase

● These findings suggest that the different ErbB4 CYT isoforms have both quantitative and qualitative signaling differences

● This may explain some of the controversy that currently prevails about cellular responses stimulated by ErbB4