cIAP1 regulates the EGFR/Snai2 axis in triple-negative breast cancer cells

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Abstract
Inhibitor of apoptosis (IAP) proteins constitute a family of conserved molecules that regulate both apoptosis and receptor signaling. They are often deregulated in cancer cells and represent potential targets for therapy. In our work, we investigated the effect of IAP inhibition in vivo to identify novel downstream genes expressed in an IAP-dependent manner that could contribute to cancer aggressiveness. To this end, immunocompromised mice engrafted subcutaneously with the triple-negative breast cancer MDA-MB231 cell line were treated with SM83, a Smac mimetic that acts as a pan-IAP inhibitor, and tumor nodules were profiled for gene expression. SM83 reduced the expression of Snai2, an epithelial-to-mesenchymal transition factor often associated with increased stem-like properties and metastatic potential especially in breast cancer cells. By testing several breast cancer cell lines, we demonstrated that Snai2 downregulation prevents cell motility and that its expression is promoted by cIAP1. In fact, the chemical or genetic inhibition of cIAP1 blocked epidermal growth factor receptor (EGFR)-dependent activation of the mitogen-activated protein kinase (MAPK) pathway and caused the reduction of Snai2 transcription levels. In a number of breast cancer cell lines, cIAP1 depletion also resulted in a reduction of EGFR protein levels which derived from the decrease of its gene transcription, though, paradoxically, the silencing of cIAP1 promoted EGFR protein stability rather than its degradation. Finally, we provided evidence that IAP inhibition displays an anti-tumor and anti-metastasis effect in vivo. In conclusion, our work indicates that IAP-targeted therapy could contribute to EGFR inhibition and to the reduction of its downstream mediators. This approach could be particularly effective in tumors characterized by high levels of EGFR and Snai2, such as triple-negative breast cancer.

Introduction
Triple-negative breast cancers (TNBCs) are characterized by the lack of estrogen receptor (ER), progesterone receptor (PR), and HER2 expression, and account for about 15% of all invasive breast cancers [1]. TNBC patients are treated with chemotherapy, usually doxorubicin and taxanes, but do

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not benefit from endocrine or HER2-directed therapy [1]. Moreover, few intervention opportunities are currently available for the many patients who develop metastatic recurrences. About 80% of TNBCs are defined basal-like according to their gene expression profiles which are reminiscent of breast basal or myoepithelial cells. From an
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Treatment with SM83 reduces the expression of Snai2 both in vivo and in vitro. a NOD/SCID mice engrafted subcutaneously with $5 \times 10^6$ MDA-MB231 were treated with intraperitoneal injection of SM83 (5 mg/kg, 5 times/week) or left untreated (4 mice/group) until the end of the experiment. Schedule of the experiment (upper panel) and tumor volumes (bottom panel) measured twice a week (significant differences in days 24, 27, and 30. *$P = 0.0476$, 0.0391, and 0.0344, respectively. Unpaired two-tailed $t$-test). b Six hours after the last injection, mice were killed, nodules collected, and analyzed by western blot to detect the levels of SM83 targets cIAP1, cIAP2, and XIAP. Actin and vinculin are shown as loading controls. c MDA-MB231 cells transfected in vitro with two siRNAs targeting cIAP1 were treated or not with 100 nM SM83 for 1 h. Western blots were performed to evaluate the levels of cIAP1, cIAP2, and XIAP 72 h after transfection. Values show the fold levels of XIAP. d Differentially expressed genes: heat map showing the 50 genes significantly upregulated and the 15 downregulated by SM83 in MDA-MB231 nodules collected as described in Fig. 1a. e Wound-healing experiments performed with MDA-MB231 cells transfected with control (NT1) or Snai2-specific siRNAs (n = 4, **$P = 0.0033$. Unpaired two-tailed $t$-test). The graph shows the percentage of gap closure after 24 h of migration. The complete experiment with the other siRNA tested is shown in Fig. S2a. f The levels of Snai2, downregulated in the GEP shown in Fig. 1d, and LRIG1, upregulated, were evaluated by western blot performed with lysates of MDA-MB231 nodules. Values show the fold levels of cIAP1 were both sufficient to induce XIAP downregulation of 50% (SMs), has been designed to target cellular IAP1 (cIAP1), cIAP2, and x-linked IAP (XIAP) [27–29]. These compounds increase the cytotoxic activity of traditional chemotherapy and prevent IAP-mediated activation of several signaling pathways [30]. We have previously demonstrated that SM83, a bivalent SM developed by us, can efficiently deplete cIAP1 and cIAP2 both in vitro and in vivo [29, 31]. By exploiting this molecule, we demonstrate here that cIAP1 is a novel regulator of EGFR expression and signaling. Moreover, we show that cIAP1 inhibition prevents EGFR-dependent expression of Snai2 and therefore the targeting of this IAP represents a new approach to reduce the aggressiveness of tumors characterized by high levels of EGFR and Snai2, such as TNBCs.

Results

SM83 treatment affects gene expression of MDA-MB231 primary tumors

To identify genes that are transcriptionally regulated by IAPs in vivo, we treated NOD/SCID mice bearing subcutaneous triple-negative breast MDA-MB231 tumors with the pan-IAP inhibitor SM83 (Fig. 1a, upper panel) and found that tumor growth was reduced by about 50% (Fig. 1a, bottom panel). We then tested the efficacy of the treatment at molecular level by evaluating the levels of the known targets of SMs and confirmed that SM83 reduced the levels of cIAP1, cIAP2, and XIAP (Fig. 1b). As SMs are known to trigger the degradation of cIAP1 and cIAP2, but not of XIAP, we investigated the potential mechanism underlying the reduction of XIAP protein levels and concluded that this is likely a consequence of cIAP1 downregulation, rather than being a direct effect of SM83 treatment. In fact, in vitro treatment with SM83 and silencing of cIAP1 were both sufficient to induce XIAP downregulation in MDA-MB231 cells (Fig. 1c).
being upregulated, and 15 downregulated (Fig. 1d). As expected, GSEA analysis revealed that SM83 treatment perturbed the expression of genes playing a role in NF-kB regulation or being targets of this pathway (Fig. S1). We focused on the 15 genes downregulated by SM83 and studied the effect of knocking down each of these genes in

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Snai2 levels are determined by cIAP1, but not by cIAP2 or XIAP, expression

As SM83 targets different IAPs, we investigated which of the known targets was responsible for SM83-mediated reduction of Snai2 levels. For this reason, cIAP1 and cIAP2 (Fig. 2a) were silenced in MDA-MB231 cells, while XIAP did not affect Snai2 levels, whereas depletion of cIAP2 had no effect. Silencing of XIAP did not affect Snai2 levels in MDA-MB231 cells, while it slightly reduced Snai2 levels in BT549 cells (Fig. 2b), although to a lesser extent compared to cIAP1. Of note, cIAP1 silencing did not affect XIAP levels in BT549 cells in contrast to what found in MDA-MB231 cells (Fig. 1c). The reduction of Snai2 mediated by cIAP1 silencing was further confirmed by using two different siRNAs and employing a number of breast cancer cell lines expressing detectable levels of Snai2 (Fig. 2c). In all the cell lines tested, cIAP1 silencing reduced Snai2 levels, confirming a widely valid regulation of Snai2 by this IAP. Nonetheless, we considered the possibility that Snai2 downregulation could be caused by a toxic effect of IAP targeting. In fact, a small percentage of cancer cell lines are known to be killed by SMs in monotherapy. Therefore, the cell lines transfected in Fig. 2c were treated with 100 nM SM83, finding that MDA-MB231 and BT549 cells were the only ones sensitive to the treatment (Fig. 2d) at this concentration. So, the latter two cell lines were pre-treated with the pan-caspase inhibitor z-VAD, which, however, was not able to prevent the SM83-dependent downregulation of Snai2 (Fig. 2e), suggesting that this reduction is a direct effect of cIAP1 inhibition and not a side effect of toxicity.

Snai2 expression is stimulated by EGFR in a MAPK-dependent manner

As IAPs are apical regulators of several pathways, we questioned which of these pathways were responsible for Snai2 regulation by using specific inhibitors for PI3K, AKT, MEK, and p38. The MEK inhibitor UO126 strongly and reproducibly reduced the expression of Snai2 in all cell lines tested (Fig. 3a) and the same effect was obtained upon ERK1 and ERK2 silencing (Fig. 3b). Importantly, Snai2 is more expressed in basal-like breast cancer cells (Fig. 3c), which are often characterized by increased levels of EGFR, and a correlation exists between EGFR and Snai2 expression in several cancer types, among whom breast cancer (Fig. 3d). Hence, we focused on the EGFR/MAPK pathway and stimulated EGFR with two of its known ligands, EGF and TGFα. As expected, the stimulation increased the expression of Snai2 (Fig. 3e) and this was attenuated by pre-treatment with the EGFR-specific inhibitor cetuximab, which reduced EGFR and ERK1/2 activation, and eventually resulted in limited EGFR-dependent stimulation of Snai2 (Fig. 3f, g). Notably, increased levels of Snai2 were also seen in normal mammary epithelial cell lines upon EGFR stimulation or in the presence of constitutive expression of active EGFR mutant (Fig. 3h), suggesting that aberrant activation of EGFR can promote Snai2 expression.

Depletion of cIAP1 prevents EGFR-dependent activation of ERK1/2 and expression of Snai2

To elucidate the role of cIAP1 in EGFR regulation and ERK1/2 activation, experiments were performed in MDA-MB231 (Fig. 4a) and BT549 (Fig. 4b) cells silenced for cIAP1 and stimulated with EGF. Depletion of cIAP1 strongly reduced ERK1/2 phosphorylation (Fig. 4a, b), and prevented the accumulation of Snai2 upon EGF or TGFα exposure (Fig. 4c). Snai2 induction was also equally repressed in normal mammary epithelial cells bearing wild-type and mutated EGFR (Fig. 4d). Finally, we checked...
whether cIAP1 knockdown affected Snai2 accumulation by inhibiting its transcription, as observed in vivo with SM83 treatment, and found that cIAP1 silencing not only reduces the basal levels of Snai2 mRNA, but also prevents its EGFR-dependent increase, both in breast cancer (Fig. 4e) and normal mammary epithelial cells (Fig. 4f).
Knockdown of cIAP1 reduces EGFR levels

We then investigated the relationship between cIAP1 and EGFR signaling. In both cancer and normal mammary epithelial cell lines, cIAP1 depletion resulted in a marked drop of EGFR levels (Fig. 5a, b), which further decreased upon stimulation with EGF. Moreover, the ligand-induced EGFR foci detected in control cells were abrogated upon cIAP1 knockdown (Fig. 5c), suggesting an impaired receptor signal transduction. As LRIG1, a known EGFR inhibitor, is upregulated in cells treated with SM83 (Fig. 1d, f), we verified whether it plays a role in cIAP1-dependent regulation of EGFR. In cIAP1-silenced BT549 cells, LRIG1 levels increased both at transcriptional (Fig. 5d) and protein level (Fig. 5e), but, in spite of this, EGFR levels only marginally increased upon LRIG1 depletion (Fig. 5f). Moreover, the transcriptional upregulation of LRIG1 was not evident after cIAP1 knockdown in MCF10A cells (Fig. 5g), which however strongly reduced EGFR levels (Fig. 5b). Taken together, these findings lead us to conclude that LRIG1 upregulation may not be the only factor responsible for EGFR inhibition.

Depletion of cIAP1 favors EGFR protein stability, but reduces its transcription

Having shown that the absence of cIAP1 reduces the levels of EGFR (Fig. 5a, b), we checked whether cIAP1 directly promotes EGFR stability or whether it affects the capacity of c-Cbl, another ubiquitin ligase known to ubiquitinate EGFR, to degrade this receptor. In fact, both cIAP1 and c-Cbl interact with EGFR as shown by proximity ligation assay (PLA) and by immunoprecipitation, respectively (Fig. 6a, b). However, cIAP1 depletion surprisingly did not reduce the stability of EGFR, but rather it increased its half-life as demonstrated by blocking protein synthesis with cycloheximide (Fig. 6c). Accordingly, the silencing of cIAP1 slightly reduced the levels and interaction of c-Cbl with EGFR (Fig. 6d), likely contributing to its increased stability. Concordant with this, overexpression of c-Cbl counteracted this effect and reduced the levels of EGFR (Fig. 6e, f) only in cells silenced for cIAP1.

Therefore, we hypothesized that cIAP1 could regulate EGFR transcription levels. Cells were transfected with cIAP1 siRNAs and analyzed by real-time PCR, which confirmed a significant downregulation of EGFR mRNA compared to controls (Fig. 6g). Moreover, as cIAP1 is known to regulate the NF-κB pathway, we investigated whether the latter is responsible for EGFR gene expression and therefore silenced three NF-κB transcription factors. In agreement with our hypothesis, the silencing of p65/RelA resulted in a reduction of EGFR protein levels (Fig. S3).

The depletion of IAPs prevents EGFR signaling independently of its downregulation

Having observed a reduction of EGFR levels upon cIAP1 silencing, we investigated the effect of IAP targeting in cells treated with SM83. To this end, we focused on SUM159 and MCF10A cells (Fig. 7a, b), which are completely resistant to SM83 treatment in terms of viability (Figs. 2d, 7c). SM83 pre-treatment had no effect on EGFR levels in SUM159 cells (Fig. 7a), and resulted only in a modest, if any, reduction of this receptor in MCF10A cells (Fig. 7b), contrarily to what observed when cIAP1 was silenced for 72 h (Fig. 5b). Nonetheless, the inhibition of IAPs massively prevented the EGF-dependent activation of ERK1/2 and the upregulation of Snai2 in both cell lines (Fig. 7a, b). The effect on ERK1/2 activation was yet minor in MDA-MB157 cells (data not shown), another cell line resistant to SM83 (Fig. 2d). We therefore checked whether the ectopic expression of EGFR could be sufficient to prevent the effect of IAP-targeting in BT549 cells, in which the downregulation of EGFR was evident upon cIAP1 silencing (Fig. 5a). Therefore, BT549 cells stably expressing ectopic EGFR were silenced for cIAP1 or pre-treated with SM83, and then stimulated with EGF. The silencing of cIAP1 prevented the upregulation of Snai2 (Fig. 7d), and the same result was confirmed by SM83-mediated targeting of cIAP1 (Fig. 7e), which also reduced the activation of ERK1/2. Therefore, IAPs, and in particular cIAP1, control the signaling of EGFR even independently of the reduction of its levels. In light of these findings, we analyzed the tumors collected from NOD/SCID mice 6 h after the last SM83 injection (Fig. 1b–f). In our GEP, EGFR was...
not found among the downregulated genes in vivo, and accordingly EGFR protein levels were not affected by SM83 treatment (Fig. 7f). Nonetheless, ERK1/2 phosphorylation was reduced by the treatment, further supporting the idea that also in vivo ERK1/2 is regulated by IAPs even when EGFR levels are not affected.

**The targeting of IAPs results in anti-tumor and anti-metastasis effect**

Finally, we evaluated the overall effect of IAP-targeting on metastasis formation. In fact, as MDA-MB231 xenografts are known to spontaneously metastasize to lungs, NOD/SCID mice were treated for 3 weeks with SM83 and then lungs were collected after a further 2 weeks (Fig. 7a, upper panel). In accordance to what had been previously observed (Fig. 1a), SM83 treatment delayed the growth of primary tumors (Fig. S4a) and also displayed an anti-metastatic effect (Fig. 8a, bottom panel), resulting in a significant reduction of both number (Fig. 8b) and size (Fig. 8c) of lung metastases. The inhibitory effect of SM83 on the growth of primary tumors and their metastasizing potential was confirmed in additional experiments also employing different administration routes (Fig. S4a–c). Of note, the effect of SM83 was transient and, at the end of the experiments, 2 weeks after the last injection with SM83, cIAP1, Snai2, and phosphorylated ERK1/2 levels were comparable in the primary tumors of either treated or untreated mice (Fig. 8d).
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**Figure a**

EGF, h: 0 0.5 3 6 0 0.5 3 6
Snai2
EGFR
pEGFR
ciAP1
pERK 1/2
ERK 1/2
Actin

**Figure b**

EGF, h: 0 0.5 3 6 0 0.5 3 6
EGFR
ciAP1
Snai2
Actin

**Figure c**

siCt:
EGFR
DAPI
siCiAP1:

**Figure d**

BT549

**Figure e**

EGF, 3h: - + - +
Snai2
ciAP1
LRIG1
Actin

**Figure f**

siCt:
EGF, 3h: - + - + - +
EGFR
LRIG1
Actin

**Figure g**

MCF10A

**Figure h**

LRIG1 expression fold change

Unstimulated  EGF 3 h
Fig. 5 Silencing of cIAP1 reduces EGFR levels. a BT549 and b MCF10A cells were transfected with control or cIAP1-specific siRNAs before being serum-starved overnight, stimulated with 20 ng/ml EGF and analyzed by western blot to detect the indicate proteins. c BT549 cells were transfected with control or cIAP1-specific siRNAs and, after 48 h, cells were serum-starved for 24 h and then stimulated 30 min with 20 ng/ml EGF. Fixed cells were incubated with anti-EGFR antibody and nuclei stained with DAPI. d LRIG1 expression levels were evaluated by real-time PCR in BT549 cells serum-starved and stimulated with 20 ng/ml EGF after transfection with control or cIAP1-directed siRNAs. Unstimulated siCtr vs. sicIAP1 *P = 0.0175, EGF 3 h siCtr vs. sicIAP1 *P = 0.0341; n = 3; two-tailed paired t-test. e Levels of Snai2 and LRIG1 in BT549 cells silenced for cIAP1 and stimulated or not with 20 ng/ml EGF. f EGFR levels in BT549 cells silenced for cIAP1 and LRIG1, stimulated or not with 20 ng/ml EGF. g LRIG1 expression levels were evaluated by real-time PCR in MCF10A cells serum-starved and stimulated with 20 ng/ml EGF after transfection with control or cIAP1-directed siRNAs. ns not significant; n = 4; two-tailed paired t-test.

Discussion

Basal-like breast cancer cells are usually characterized by high expression of the EGFR receptor [2], which is a major regulator of several signaling pathways that sustain cancer cell survival and dissemination. Moreover, EGFR, through the engagement of the MAPK cascade, induces the expression of the EMT-associated factor Snai2 [7, 9]. Here, we show that cIAP1 represents a novel regulator of EGFR by promoting EGFR signaling and expression, while simultaneously favoring its protein degradation. Hence, the targeting of cIAP1 strongly reduces EGFR signaling with consequent inhibition of Snai2 expression, which is a well-known promoter of breast cancer cell aggressiveness (Fig. S5).

Together with their anti-apoptosis activity, IAPs play a pivotal role in the regulation of receptor-mediated signaling [32] being components of TNF-receptor superfamilly members [33, 34] and pattern recognition receptors [35]. Accordingly, small molecules designed to inhibit IAPs and trigger cancer cell death also perturb several signaling pathways such as MAPK and NF-kB cascades [30] and modify the expression of a plethora of genes [36, 37]. Accordingly, the in vivo administration of SM83, a SM designed and characterized in our laboratory [29, 31], significantly modulated 65 genes in primary MDA-MB231 nodules. In agreement with other studies, the majority of the perturbed genes are known to be expressed in a NF-kB-dependent manner [36].

Overall, the targeting of IAPs by SM83 resulted in an anti-tumor and anti-metastatic effect. Many different mechanisms could have contributed to this outcome, including direct killing of the cancer cells, effect on the microenvironment [31] and the associated blood vessels [38], as well as perturbation of gene expression. We employed unbiased approaches, which underscored Snai2 as one of the most downregulated genes and showed that its reduction hinders cell motility and, partially, cell proliferation. Snai2 expression was inhibited both in vivo and in vitro by SM83, and, through additional experiments, we identified cIAP1 as the determinant of Snai2 expression. In fact, we provided evidence that the targeting of cIAP1 hinders EGFR signaling by reducing the levels of this receptor and also by preventing the EGFR-mediated activation of ERK1/2. This observation could be clinically relevant as SM treatment might be particularly effective in cancer cells expressing high levels of EGFR, such as breast cancer basal-like cells, in combination with EGFR-targeted therapy [4]. Moreover, IAP-targeting could represent an indirect approach to inhibit Snai2 for which only proof-of-concept molecules [39] or compounds directed against its interactors have been described so far [24, 40]. Inhibition of Snai2 is considered a promising approach to prevent cancer metastasis and resistance to therapy, and there is considerable evidence showing that Snai2-targeting reduces the capability of breast cancer cells to migrate and disseminate to distal organs by reducing their stem-like properties and EMT features [22]. However, further work is still necessary to clarify the function of EMT in metastatisation and the precise role of Snai2 in this process. In fact, recent works have questioned the importance of EMT in metastasis formation [41], also proposing a negligible role of Snai2 in cancer cell metastatic properties [42].

Notably, only a small percentage of cancer cell lines are killed by SMs in monotherapy [43]. Cell death is triggered in a TNF-dependent manner, but cells can acquire resistance to SMs by upregulating LRIG1 [44]. This ubiquitin-ligase is an intestinal stem cell marker and negatively regulates several receptors [45], including Met and EGFR [46]. Therefore, we first investigated the hypothesis that LRIG1 upregulation was responsible for EGFR reduction upon cIAP1 depletion, but paradoxically the protein stability of this receptor was increased by cIAP1 silencing. Therefore, we considered the possibility that other ubiquitin-ligases could regulate EGFR stability and studied the effect of c-Cbl [47] and cIAP1 itself. The latter has recently been shown to regulate the signaling of EGFRvIII variant [48]. Interestingly, cIAP1 depletion increased EGFR stability, supporting the notion that cIAP1 promotes EGFR degradation likely as a consequence of its efficient signaling and internalization. Accordingly, cIAP1 silencing reduced the levels of c-Cbl, which is known to ubiquitinate EGFR and promote its degradation [49, 50]. This effect could contribute to EGFR stabilization and, in support to this hypothesis, c-Cbl ectopic expression counteracted the effect of cIAP1 silencing and restored EGFR degradation. Despite the increased stability of EGFR, the overall effect of cIAP1 targeting resulted in a strong reduction of the receptor levels in a few cell lines. Our data support the notion that in these cell lines cIAP1 favors EGFR gene
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expression, and most likely in a NF-kB-dependent manner. Ultimately, cIAP1-depletion-dependent reduction of EGFR could impair MAPK activation and, in turn, reduce the expression of the downstream gene Sna12. Of note, the downregulation of EGFR appeared dispensable for the inhibition of ERK1/2 activation and Sna12 expression upon cIAP1 depletion. In fact, this effect was observed also when depletion of IAPs did not result in a significant reduction of EGFR levels as demonstrated by several lines of evidence. First, when MCF10A and SUM159 cells were exposed to SM83 for a period of time sufficient to deplete cIAP1, but not to reduce EGFR protein levels, ERK1/2 phosphorylation and Sna12 stimulation were still prevented even upon EGFR stimulation. In line with this evidence, EGFR ectopic expression was not sufficient to restore ERK1/2 and Sna12 expression in BT549 cells treated with SM83 or silenced for cIAP1. Lastly, MDA-MB231 tumors collected from NOD/SCID mice revealed that SM83 treatment did not affect EGFR levels, but profoundly reduced ERK1/2 activation.

Overall, this work demonstrates that cIAP1 promotes EGFR cascade by sustaining the receptor expression levels and by allowing its downstream signaling, and this effect eventually results in Sna12 expression. Therefore, our work provides new information regarding the mechanisms underlying EGFR regulation and the rationale for combining EGFR inhibitors with IAP-directed small molecules. This approach could be particularly effective in reducing the aggressiveness of cancer cells characterized by the pathological activation of EGFR signaling pathways or high levels of this receptor, as the case of basal-like cells which represent the majority of TNBCs.

Materials and methods

Cell culturing and reagents

The human breast adenocarcinoma cell lines MDA-MB231, BT549, MDA-MB157, SkBr3, and HCC1937 cell lines (from American Type Culture Collection) were cultured in vitro with RPMI-1640 medium (Lonza Group, Basel, CH) supplemented with 10% fetal bovine serum (FBS; EuroClone, Milan, IT), 2 mM glutamine, sodium pyruvate, and non-essential amino acids (LONZA). SUM149 and SUM159 (Asterand Bioscience, Detroit, MI), together with the human hTERT-immortalized human mammary epithelial cell line HME—parental and bearing the EGFR delE746A750 mutation (HME EGFR hereafter)—and the human mammary epithelial MCF10A—parental and bearing the EGFR delE746A750 mutation (MCF10A EGFR hereafter)—were cultured as already described [51] in DMEMF-12 ( Gibco), supplemented with 10% FBS, 2 mM glutamine, 20 ng/ml EGF (Cat. #GRF-10544, Selleck Chemicals, Munich, D), 10 µg/ml insulin (Sigma-Aldrich), 500 µg/ml hydrocortisone (Sigma-Aldrich), BT474, MDA-MB453, T47D, and MDA-MB361 cell lines were maintained in DMEM ( Gibco) supplemented with 10% FBS and 2 mM glutamine. All cells were grown at 37 °C in a humidified incubator with 5% CO2. TGFOX (#100–16A) was purchased from Peprotech (London, UK), while cycloheximide (CHX, #239764) from Calbiochem. For specific stimulation of EGFR, cells were serum-starved overnight before being stimulated with the indicated concentrations of EGF and TGFOX in medium supplemented with 0.1% FBS. HEK293FT (Thermo Fisher Scientific, Milan, IT) for lentiviral production were cultured in DMEM with 10% FBS. Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and Lipofectamine RNAiMAX (Thermo Fisher Scientific) reagents were used for plasmid and siRNA transfections, respectively. For silencing experiments, cells were reverse transfected as already described [51]. Cells were treated with the PI3K and AKT (LY294002 and Tri-ciribine, Enzo Life Sciences, Plymouth Meeting, PA), MEK (U0126, Calbiochem, Merck KGaA, Darmstadt, D), and p38 (SB203580, Selleckchem, USA) inhibitors. Cetuximab was provided by the pharmacy of the Istituto Nazionale dei Tumori (Milan, IT). SM83 synthesis has been described elsewhere [29]. Z-VAD(OMe)-FMK was purchased by BIOMOL. Cell viability was measured 24 h after treatment with SM83 by using the CellTiter-Glo Luminescent Cell Viability Assay according to the manufacturer’s protocol (Promega).

In vivo experiments

Experiments were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori.
(INT) of Milan according to institutional guidelines and by the Italian Minister of Health (Projects INT_12_2011 and INT_02_2015). Mice were maintained in laminar flow rooms keeping temperature and humidity constant and had free access to food and water. NOD/SCID mice were subcutaneously engrafted with $5 \times 10^6$ MDA-MB231 cells and,
after 2 weeks, were treated with 5 mg/kg SM83 injected intraperitoneally or intravenously 5 times/week for 3 weeks. Tumor growth was evaluated by biweekly measurements of tumor diameters with a Vernier caliper and tumor volume (TV) was calculated according to the formula: \( \frac{1}{2} \times l \times w \times h \) where \( l \), \( w \), and \( h \) are length, width, and height, respectively. A good correlation was found between TVs predicted with this formula and actual tumor weight measured post-mortem (Fig. S4d). Animals were killed 6 h after the last injection and tumors collected for biochemical and gene expression analysis. In metastasis studies, NOD/SCID mice were killed 2 weeks after the last SM83 administration and, together with subcutaneous nodules, lungs were collected and formalin-fixed/paraffin-embedded for IHC detection of metastasis with anti-human vimentin antibody (M0725, DAKO).

**Gene expression profiling**

GEP was performed by the Functional Genomics and Bioinformatics Core Facility Illumina of INT by using the Human HT-12_V4_0_R2 platform (tot. 47323 probes). Analysis was performed through R 2.15.2, with “lumi” and “limma” packages from Bioconductor. Only probes with Absolute Fold Change >1.5 and FDR <0.05 were considered significant. Expression profiles are deposited in the Gene Expression Omnibus Repository, GSE98691 accession number.

**Gene silencing and overexpression**

FlexiTube siRNAs specific for cIAP1 (Hs_BIRC2_7 and 8), cIAP2 (Hs_BIRC2_8 and 9), ERK1 (Hs_MAPK3_3 and 7), ERK2 (Hs_MAPK1_9 and 10), Nfk-B2 (Hs_NFKB2_1), NF-kB1 (Hs_NFKB1_3), RELA (Hs_RELA_5), LRIG1 (Hs_LRIG1_5 and 6) were purchased from Qiagen (Valencia, CA, USA). The control siRNA (siCtr) was synthesized by Eurofins Genomics (sequence 5’-CGUACCGGAAUAUCUGATT-3’), while siGENOME SMARTpool for XIAP (M-004098-01), Sna2 (M-017386-00) and its control, siGENOME Non-Targeting siRNA Pool #1 (NT1; D-001206-13), were from Dharmacon (GE Healthcare, Lafayette, CO, USA).

To ectopically express EGFR and c-Cbl, cells were transfected with lentiviral particles prepared by HEK293FT packaging cells as already described [52]. The plasmid for human EGFR (Myc/Flag-tagged) ectopic expression was purchased by Origene Company (#RC217384L1; Rockville, MD, USA), while the c-Cbl-expressing vector (RRL-CMV-CBL) was kindly provided by Prof. Pier Paolo Di Fiore. To overexpress cIAP1, cells were transfected with pcDNA3.1 plasmids [53] kindly provided by Prof. Jon Ashwell (Addgene plasmid # 8311).

**Western blot and immunoprecipitations**

For western blot analysis, cells were harvested, washed twice with ice-cold PBS, and boiled for 10 min in lysis buffer (125 mM Tris HCl, pH 6.8, 5% sodium dodecyl sulfate, SDS) and then added with protease inhibitors. Samples were sonicated, clarified at room temperature by centrifugation for 15 min at 11,000 rpm and then quantified with microBCA (Euroclone). Cleared supernatants were separated by SDS-PAGE on precast 4–12% Bis-Tris NuPAGE gels (Thermo Fisher Scientific) and proteins were transferred to PVDF membranes (Merck Millipore, Darmstadt, Germany) using the XCell II blot module (Thermo Fisher Scientific). Membranes were then saturated for 30 min in Tris-buffered saline containing 4% BSA and incubated overnight with the anti-human primary antibodies recognizing cIAP1 #ab108361, total EGFR #ab30 (Abcam); Sna2 #9585, LRIG1 #12752, NF-kB2 #4882, NF-kB1 #3035, Cleaved Caspase3 #9501, p65/RelA #3034, pEGFR Tyr1068 #2236, c-Cbl #2747, Myc-Tag #2278 (Cell Signaling Technology, Danvers, MA, USA); phospho-ERK1/2 #4370, ERK1/2 #4695, Erk1 #312, Erk2 #3131, Akt #9272, pAkt #4060, EGFR #2775, c-Myc #9402, p65p65/RelA #3034, pERK1/2 #4370, ERK1/2 #4695, Erk1 #312, Erk2 #3131, Akt #9272, pAkt #4060, EGFR #2775, c-Myc #9402, p65/RelA (Sigma-Aldrich); cIAP2 #552783 and XIAP #610763 (BD Biosciences). After incubation with the proper secondary antibody (anti-rabbit and anti-mouse IgG were purchased from GE Healthcare, UK, and anti-goat IgG from Thermo Fisher Scientific; all secondary antibodies were diluted 1:2000), proteins were detected by chemiluminescence (EuroClone).

For FLAG-tagged EGFR immunoprecipitations, BT549 cells were lysed in E1A lysis buffer (ELB) buffer (150 mM NaCl, 50 mM Heps, pH 7.5, 5 mM EDTA, 0.5% NP40) at 4°C for 30 min and 1 mg of total protein extract was incubated at 4°C for 3 h with Flag-M2 Affinity Gel (#A2220, Sigma-Aldrich) previously washed with ELB...
cIAP1 regulates the EGFR/Snai2 axis in triple-negative breast cancer cells

(a) Lung metastasis

Untreated

SM83

Anti Vimentin

(b) Lung metastasis number

Lung metastasis size

(d) Western blot analysis of clAP1, Vinculin, Snai2, EGFR, pERK1/2, ERK1/2, and Vinculin in untreated and SM83-treated samples.
buffer three times. The bound polypeptides were analyzed by SDS-PAGE and immunoblotting.

**Migration assay**

MDA-MB231 cells were reverse transfected in Ibidi inserts (4 x 10^4 cells/chamber) and motility tested 72 h later in wound-healing experiments performed as already described [54]. Image acquisition and data analysis were carried out with a Cell-IQ instrument (CM-Technologies) and the integrated software.

**RNA extraction and real-time PCR**

For real-time PCR, total RNA was extracted from cells with the miRNeasy mini columns (Qiagen) and 1 μg RNA was reverse-transcribed using the Transcriptor First Strand cDNA Synthesis kit (Roche, Indianapolis, IN, USA). Probes for human SNAI2 (Hs_00950344_m1), LRIG1 (Hs_00394267_m1), EGFR (Hs_01076090_m1), and GAPDH (Hs_00394267_m1) were purchased from Applied Biosystems (Foster City, USA). Real-time PCR was carried out in triplicate using TaqMan Master Mix (Applied Biosystems) and samples were analyzed through the ViiA 7 Real-Time PCR System (Applied Biosystems). All transcript levels were normalized to that of GAPDH.

**Immunofluorescence and PLA**

Cells were fixed for 10 min with 4% paraformaldehyde, permeabilized for 10 min at room temperature with 0.2% Triton X-100 and blocked in PBS, 3% BSA, 0.1% Tween-20. Cover slips were then incubated overnight at 4 °C in a humidified chamber with antibodies directed to EGFR (mouse, dilution 1:200, abcam). Fluorescence images were acquired using a fluorescence microscopy and digital image acquisition on a Nikon Eclipse E1000 equipped with a DS-U3 CCD camera. For PLA assay, cover slips were stained with anti EGFR (mouse, dilution 1:200, abcam) and/or cIAP1 (rabbit, dilution 1:2000, abcam). After the overnight incubation with primary antibodies, cells were stained with the PLA probes diluted 1:5 in antibody diluent (Sigma-Aldrich) in a humidified chamber for 1 h at 37 °C. Subsequent hybridization, ligation, amplification, and detection were performed as described in the manufacturer’s protocol (Duolink In Situ PLA; Sigma-Aldrich).

**Graphs, statistical analysis, and image analysis**

Graphs and statistical analysis was performed using GraphPad Prism 5.02. A value of P < 0.05 was considered statistically significant. Western blot densitometric analysis was performed with ImageQuant 5.2. Graphs showing the average of different experiments are shown in Supplementary Fig. S6. GEP analyses were performed as previously described.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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