

1 **ITCH-dependent proteasomal degradation of c-FLIP induced by the anti-HER3**
2 **antibody 9F7-F11 promotes DR5/caspase 8-mediated apoptosis of tumor cells**

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1 **Abstract**

2
3 **Background:** HER3/ErbB3 receptor deletion or blockade leads to tumor cell apoptosis,
4 whereas its overexpression confers anti-cancer drug resistance through upregulation of
5 protective mechanisms against apoptosis. We produced the anti-HER3 antibody 9F7-F11
6 that promotes HER3 ubiquitination and degradation via JNK1/2-dependent activation of the
7 E3 ubiquitin ligase ITCH, and that induces apoptosis of cancer cells. Cellular FLICE-like
8 inhibitory protein (c-FLIP) is a key regulator of apoptotic pathways. Here, we wanted to
9 determine the mechanisms underlying the pro-apoptotic effect of 9F7-F11.

10 **Methods:** Anti-HER3 antibody-induced apoptosis was assessed by western blot, and by flow
11 cytometry measurement of Annexin V/7-AAD-labelled tumor cells (BxPC3, MDA-MB-468 and
12 DU145 cell lines). c-FLIP/ITCH interaction and subsequent degradation/ubiquitination were
13 investigated by co-immunoprecipitation of *ITCH*-silenced vs scramble control cells. The
14 relationship between ITCH-mediated c-FLIP degradation and antibody-induced apoptosis
15 was examined by western blot and flow cytometry of tumor cells, after *ITCH* RNA
16 interference or by pre-treatment with ITCH chemical inhibitor chlorimipramine (CI).

17 **Results:** Following incubation with 9F7-F11, cancer cell apoptosis occurs through activation
18 of caspase-8, -9 and -3 and the subsequent cleavage of poly(ADP-ribose) polymerase
19 (PARP). Moreover we showed that ubiquitination and proteasomal degradation of the anti-
20 apoptotic protein c-FLIP was mediated by USP8-regulated ITCH recruitment. This effect was
21 abrogated by *ITCH*- and *USP8*-specific RNA interference (siRNA), or by the ITCH chemical
22 inhibitor CI. Specifically, *ITCH* silencing or CI blocked 9F7-F11-induced caspase-8-mediated
23 apoptosis of tumor cells, and restored c-FLIP expression. *ITCH*-silencing or CI concomitantly
24 abrogated HER3-specific antibody-induced apoptosis of Annexin V/7-AAD-labelled BxPC3
25 cells. 9F7-F11 favored the extrinsic apoptosis pathway by inducing TRAIL-R2/DR5
26 upregulation and TRAIL expression that promoted the formation of death-inducing signaling
27 complex (DISC), leading to caspase-8-mediated apoptosis. Incubation with 9F7-F11 also
28 induced BID cleavage, BAX upregulation and BIM expression, which initiated the caspase-

1 9/3-mediated mitochondrial death pathway. The anti-HER3 antibody pro-apoptotic effect
2 occurred concomitantly with downregulation of the pro-survival proteins c-IAP2 and XIAP.

3 **Conclusions:** The allosteric non-neuregulin competing modulator 9F7-F11, sensitizes tumor
4 cells to DR5/caspase-8-mediated apoptosis through ITCH-dependent downregulation of c-
5 FLIP.

6 **Keywords:** Cancer, HER3, antibody, apoptosis, c-FLIP, ITCH/AIP4

7

1 **Background**

2

3 Apoptosis is a cell death type that is essential during embryonic development and for
4 homeostasis maintenance. It is also one of the processes deregulated during cell
5 transformation. In many cancers, activation of the HER3 (ErbB3) receptor frequently results
6 in strong and aberrant activation of the AKT pathway, a critical oncogenic stimulus that leads
7 to apoptosis resistance. HER3 knockdown [1] or pharmacological blockade [2] directly
8 restores tumor cell-specific apoptosis. Conversely, HER3 overexpression confers drug
9 resistance to paclitaxel in breast cancer via upregulation of survivin, a pro-survival protein
10 that inhibits apoptosis [3]. In this case, targeting HER3 with antibodies indirectly counteracts
11 drug resistance by favoring tumor cell apoptosis [4].

12 Apoptosis is induced through two main pathways. The extrinsic pathway is activated by the
13 binding of specific ligands (e.g., FASL, TRAIL, and TNF) to their death receptors (DR) at the
14 cell surface (FAS/CD95, DR4/TRAIL-R1 and DR5/TRAIL-R2, TNFR1 and TNFR2) [5–7].
15 These ligand/receptor complexes recruit adaptor molecules, such as Fas-associated protein
16 with death domain (FADD), on their death effector domains (DED), leading to the formation
17 of Death-Inducing Signaling Complexes (DISC) that in turn recruit pro-caspase 8. Following
18 autocatalytic cleavage [8,9], activated caspase-8 induces cleavage and activation of
19 caspase-3, -6 and -7 directly in type I cells [10], or indirectly in type II cells, by BID cleavage
20 into the truncated form tBID [11] that activates the intrinsic apoptotic machinery by formation
21 of tBID/BAX complexes in mitochondria [12]. These complexes form mitochondrial pores for
22 cytochrome C release that promotes apoptosome formation and then caspase-9
23 cleavage/activation to induce apoptosis [13]. DISC-induced apoptosis is negatively regulated
24 by a short-lived protein called FLICE-like inhibitory protein (c-FLIP) [14], a competitive
25 mimetic of pro-caspase 8 [15–17] with two DEDs, like the initiator caspase. However, due to
26 the absence of the catalytic cysteine residue present in pro-caspase 8, c-FLIP is inactive,
27 and thus is considered to be an anti-apoptotic protein. c-FLIP also blocks the intrinsic

1 apoptotic pathway by competing with caspase-8 to prevent BID activation. c-FLIP is
2 upregulated in many cancers (e.g., pancreatic [18], breast [19], prostatic [20], and colorectal
3 [21] cancer, glioblastoma [22], Burkitt and non-Hodgkin lymphoma [23,24]). c-FLIP
4 upregulation promotes defects of DR-mediated apoptosis and resistance to several anti-
5 cancer drugs [25]. However, c-FLIP is an unstable protein with rapid turnover via
6 ubiquitination and proteasomal degradation by specific E3 ubiquitin ligases, such as ITCH
7 [26–28]. Cisplatin treatment sensitizes tumor cells to apoptosis by favoring ITCH-mediated c-
8 FLIP downregulation [29,30]. Conversely, cystatin B inhibits TRAIL-induced apoptosis in
9 melanoma cells by protecting c-FLIP from degradation by ITCH [31].

10 We previously showed that several anti-HER3 antibodies, including the allosteric non-
11 neuregulin competing modulator 9F7-F11 [32], induce ITCH-mediated HER3 degradation
12 [33] and promote apoptosis in tumor cells [2]. Among numerous anti-HER3 antibodies
13 described [34,35], 9F7-F11 is the only anti-HER3 antibody whose binding and affinity to
14 HER3 are enhanced in the presence of neuregulin 1 (NRG1) [32]. This property translated *in*
15 *vivo* into an efficient anti-tumor activity in NRG1-addicted and NRG1-rearranged cancer cells
16 [32], making it a first-in-class antibody. Here, we asked whether its pro-apoptotic effect was
17 regulated by ITCH-dependent c-FLIP expression regulation. We found that 9F7-F11
18 sensitizes tumor cells to DR5/caspase-8-mediated apoptosis through ITCH-dependent
19 downregulation of c-FLIP.

20

1 **Methods**

2

3 **Cell lines**

4 The BxPC3 (pancreas), MDA-MB-468 (breast), and DU145 (prostate) human cancer cell
5 lines were obtained from the American Type Culture collection (ATCC) (Rockville, MD), and
6 grown as recommended by ATCC. All cell lines were free of mycoplasma contamination
7 (determined by the MycoAlert™ Detection Kit (Lonza, Switzerland) and were authenticated
8 by short tandem repeat profiling using the Promega PowerPlex 21 System. These three cell
9 lines both expressed EGFR, HER2 and HER3 receptors, and the HER3 ligand neuregulin,
10 and are all equipped with the ITCH/USP8/USP9X machinery (Additional file 1: Figure S1).
11 We previously demonstrated that the anti-HER3 antibody 9F7-F11 induced HER3
12 ubiquitination and degradation in these three cell lines through a JNK-dependent ITCH
13 activation [33]. Furthermore, 9F7-F11 reduced HER2/HER3 heterodimerization, increased
14 HER3 homodimers, without affecting HER2 homodimers in HER2/HER3-transfected 3T3
15 cells [36].

16

17 **Antibodies and other reagents**

18 The anti-HER3 antibody 9F7-F11 has been described elsewhere [2,32,33,36]. Rabbit
19 monoclonal antibodies against HER3 and phosphorylated (p) HER3 (Tyr1289), PARP (clone
20 46D11), cleaved caspase-8 (Asp391; clone 18C8), caspase-9 and cleaved caspase-9,
21 caspase-3 (clone 8G10) and cleaved caspase-3, XIAP, DR5 (clone D4E7), BIM, c-FLIP
22 (clone D16A8), USP9X, β -actin and β -tubulin were from Cell Signaling Technology (Danvers,
23 MA). The mouse monoclonal antibody against ITCH was from BD Biosciences (San José,
24 CA; ref.611199). The rabbit polyclonal antibodies against HER3 (clone C17), USP8, USP9X,
25 c-FLIP_{L/S} (clone H-202), BAX, FAS, TRAIL, DcR2, cIAP2, and BID were from Santa Cruz
26 Biotechnology (Santa Cruz, CA). For detection of activated ITCH, a rabbit anti-pITCH
27 (Thr222) antibody from Millipore (Billerica, MA) was used. The human recombinant NRG1- β 1

1 extracellular domain (ECD) was from RD Systems (Minneapolis, MN) and was used at 100
2 ng/ml. The proteasome inhibitor MG132 and chlorimipramine (CI) were from Sigma-Aldrich
3 (St Louis, MO). The control IgG antibody used for co-immunoprecipitation experiments was
4 from Santa Cruz Biotechnology.

5

6 **siRNA transfections**

7 2×10^6 BxPC3 cells were plated in 10 cm-dishes with RPMI medium without antibiotics until
8 50% confluence. Cells were then transfected with 50 nM of pools of four different siRNAs
9 against human *ITCH* or *USP8*, or scramble control (ON-TARGETplus SMART pool,
10 Dharmacon, Germany) in OptiMEM medium using Interferin™ (Life Technologies, Carlsbad,
11 CA). After 4h of transfection, medium was replaced by RPMI for another 48h before using
12 the cells for apoptosis measurement by flow cytometry, or for another 72h before using the
13 cells for western blot analysis.

14

15 **Annexin V/7-AAD apoptosis measurement by flow cytometry**

16 Cell apoptosis was assessed using Annexin V/7-Amino actinomycin D (7-AAD) apoptosis kit
17 (Beckman Coulter-Immunotech, Marseille, France) according to the manufacturer's
18 instructions. Briefly, 10^5 serum-starved BxPC3 cells were transfected with 50 nM *ITCH* or
19 scramble control siRNAs, as described above. Alternatively, BxPC3 cells were pre-treated
20 with 15 μ M of *ITCH* chemical inhibitor CI. After 48h, cells were washed and treated with 50
21 μ g/ml of anti-HER3 antibody 9F7-F11, with or without 100 ng/ml of NRG1 for 96h. As positive
22 control, 300 nM staurosporine (Sigma, Saint-Louis, MO) was incubated with BxPC3 cells for
23 6-20h. After Annexin V/7-ADD labeling of treated cells, data were acquired on a Gallios flow
24 cytometer and analyzed with the Kaluza software (Beckman Coulter). All experiments were
25 performed in triplicates.

26

27

28

1 **Cell lysis and immunoprecipitation**

2 10x10⁶ BxPC3 cells were lysed in CHAPS buffer (Sigma-Aldrich) containing the protease
3 inhibitor cocktail V (Calbiochem, Billerica, MA) and the phosphatase inhibitor cocktail II
4 (Sigma-Aldrich). For c-FLIP_{LS} immunoprecipitation (Fig.4), 2 mg of each total cell lysate was
5 pre-cleared by overnight addition of 50 µl of magnetic beads (DynabeadsTM; Life
6 Technologies), to capture and remove the anti-HER3 antibody 9F7-F11. Supernatants (2 mg)
7 were then incubated with 2 µg of the anti-c-FLIP_{LS} antibody H-202, which recognizes both c-
8 FLIP_L and c-FLIP_S, at 4°C for 6h before overnight incubation with 20 µl of Dynabeads
9 magnetic beads at 4°C under agitation. Samples were washed five times with 400 µl CHAPS
10 buffer, re-suspended in 100 µl of 2X SDS Laemmli buffer and heated at 90°C for 10min
11 before electrophoresis. No c-FLIP protein was immunoprecipitated after incubation with
12 beads alone or with the control IgG antibody.

13 HER3/c-FLIP_{LS} double immunoprecipitation was performed after NRG1 stimulation and/or
14 9F7-F11 incubation of BxPC3 cells (Fig.3). First, total cell lysates (2 mg) were incubated with
15 2 µg of the anti-HER3 antibody 2F12, which recognizes the HER3 intracellular C-terminal tail
16 and does not compete with 9F7-F11. The incubation was performed at 4°C for 6h before
17 overnight incubation with 20 µl of magnetic Dynabeads at 4°C under agitation. Total
18 supernatants were recovered and then incubated with 2 µg of the anti-c-FLIP antibody H-202
19 at 4°C for 6h, before overnight incubation with 20 µl of Dynabeads magnetic beads at 4°C
20 under agitation. Samples were then processed as described above before electrophoresis.

21 DR5-DISC was isolated after immunoprecipitation (Fig.6) using the Dynabeads system, as
22 described by the manufacturer. Briefly, 2 mg of total cell lysates from 9F7-F11-treated
23 BxPC3 cells were incubated with magnetic beads previously cross-linked with the anti-DR5
24 antibody D4E7. The Dynabeads bead-coupled DISC complexes were immunoprecipitated
25 overnight at 4°C in a rotator, and then washed ten times with CHAPS buffer, before
26 electrophoresis and western blotting.

27

1 **Western blotting**

2 2×10^6 cells/dish were cultured at 37°C for 24h. After serum starvation in RPMI/1%serum with
3 antibiotics for 24h, cells were incubated with various compounds. For short kinetics, cells
4 were stimulated with 100 ng/ml of NRG1 and/or incubated with 50 μ g/ml of 9F7-F11 for
5 15min to 3h. For long kinetics, cells were incubated with 50 μ g/ml of anti-HER3 antibody
6 9F7-F11 for 24h to 120h. After incubation, cells were washed, scraped and lysed with
7 CHAPS buffer (Sigma-Aldrich), as indicated above. After washing in 1X PBS, the insoluble
8 fraction was removed by centrifugation, and protein concentration in cell lysates was
9 determined using the BCA assay. 200 μ g of protein lysates were directly mixed with Laemmli
10 buffer and heated at 95°C for 5min. After electrophoresis in reducing conditions, proteins
11 were transferred to polyvinylidenedifluoride membranes (Millipore) and then incubated with
12 the relevant primary and peroxidase-conjugated secondary antibodies, as previously
13 described [33]. β -tubulin or β -actin were used as loading control.

14

15 **Statistical analysis**

16 Apoptosis values represent the means \pm SD for at least three independent experiments
17 performed in triplicates. The significance of differences between experimental variables was
18 determined using the two-tailed Student's *t* test. The significance of *P* values are **P*<0.05,
19 ***P*<0.01 or ****P*<0.001.

20

1 **Results**

2

3 **The anti-HER3 antibody 9F7-F11 induces tumor cell apoptosis through caspase-8/9/3** 4 **activation and PARP cleavage**

5 We previously demonstrated by flow cytometry analysis after Annexin V/7-AAD staining that
6 the anti-HER3 antibodies 9F7-F11, 16D3-C1 and H4B-121 induce apoptosis of pancreatic
7 cancer cells [2]. To precisely determine the underlying molecular mechanisms, we assessed
8 apoptosis induction in three cancer cell lines at different time points during incubation with
9 the allosteric NRG1 non-competing antibody 9F7-F11 [2,32,33]. 9F7-F11 induced caspase-8
10 cleavage into the p41/43 and p18 C8c fragments in BxPC3 (pancreas), DU145 (prostate)
11 and MDA-MB-468 (breast) at 48h (Fig.1). The p37 C9c cleavage product, which indicates
12 activation of caspase-9, appeared at 24h for BxPC3 and DU145 cell lines, and 48h for MDA-
13 MB-468. p17/19 C3c cleavage products, which indicate activation of caspase-3, mainly
14 occurred 48h-post 9F7-F11 treatment for MDA-MB-498 and DU145, and 72h-post treatment
15 for BxPC3 (Fig.1). Caspase activation was followed by PARP cleavage from 24h-48h to the
16 end of the experiment (120h) (Fig.1). Incubation with the HER3 ligand NRG1 alone did not
17 induce apoptosis BxPC3 cells (Additional file 1: Figure S2). Conversely, 9F7-F11 could
18 induce apoptosis of BxPC3 cells (caspase-8/9/3 cleavage/activation and PARP cleavage)
19 also in the presence of NRG1 (Additional file 1: Figure S2). This demonstrated that 9F7-F11-
20 induced apoptosis via caspase activation is a general mechanism of cancer cell death,
21 independent of ligand stimulation.

22

23 **9F7-F11 induces c-FLIP proteasomal degradation**

24 We previously showed that 9F7-F11 promotes HER3 ubiquitination and proteasomal
25 degradation through JNK1/2-dependent ITCH activation [33]. As c-FLIP also is an ITCH
26 target [26,27], we asked whether this anti-apoptotic factor was ubiquitinated and degraded
27 concomitantly with HER3. Expression of the long isoform c-FLIP_L was notably reduced in

1 BxPC3, MDA-MB-468, and DU145 cells after 2h-incubation with 9F7-F11, but not with NRG1
2 (Fig.2a). ITCH phosphorylation was increased at 10-30min until the end of the experiment
3 (2h) (Fig.2a). Expression of USP8 and USP9X, which stabilize ITCH by preventing its auto-
4 ubiquitination [27,37], was globally stable during the entire experiment, except for BxPC3
5 cells at 2h post-9F7-F11 treatment. Both long and short c-FLIP isoforms (c-FLIP_L and c-
6 FLIP_S) and HER3 were downregulated after 3h of incubation with 9F7-F11 (Fig.2b). c-FLIP_L
7 expression, as well as HER3 expression, was reduced in BxPC3 and MDA-MB-468 cells
8 after longer incubation with 9F7-F11, but not with medium or NRG1 (Fig.2c). Then, to
9 determine whether 9F7-F11 induced c-FLIP degradation via the proteasome, we pre-
10 incubated, or not, cells with the proteasome inhibitor MG132 for 4h, before addition of 9F7-
11 F11 with or without NRG1 (Fig.2d). Pre-incubation with MG132 inhibited the 9F7-F11-
12 induced reduction in c-FLIP_L level, as previously reported for HER3 [33]. These findings
13 demonstrated that 9F7-F11 reduces substantially c-FLIP and HER3 expression via
14 proteasomal degradation, and independently of ligand stimulation.

15

16 **USP8-regulated ITCH binding to c-FLIP mediates c-FLIP ubiquitination in cancer cells** 17 **incubated with 9F7-F11**

18 To determine whether c-FLIP binds to ITCH following antibody treatment, we collected
19 BxPC3 cells at different time points during incubation with NRG1 or/and 9F7-F11, and
20 immunoprecipitated them with anti-HER3 and anti-c-FLIP antibodies. We observed ITCH co-
21 immunoprecipitation with HER3, and HER3 ubiquitination after 15min of incubation with 9F7-
22 F11 (with or without NRG1), and HER3 degradation at 2h (Fig.3, upper panel). In c-FLIP co-
23 immunoprecipitation, strong c-FLIP_L ubiquitination at 2h was correlated with stronger ITCH
24 interaction (Fig.3, middle panel). ITCH was not co-immunoprecipitated with c-FLIP_L in BxPC3
25 cells incubated with medium or NRG1 alone (Fig.3, middle panel), suggesting that ITCH
26 needs to be activated for substrate binding, as shown in HEK293 cells [38] and in our
27 previous study [33].

1 USP8 interacts and stabilizes c-FLIP by deubiquitination, leading to DR-mediated apoptosis
2 suppression [39]. USP8 also interacts with and stabilizes ITCH to induce c-FLIP
3 ubiquitination, upon AKT inhibition, leading to TRAIL-induced apoptosis [27]. We already
4 demonstrated that USP8 controls ITCH stability during 9F7-F11-induced HER3 ubiquitination
5 and degradation [33]. Here, we found that USP8 was present in the c-FLIP_L-ITCH complex in
6 untreated cells, but was slightly reduced after 2h of 9F7-F11 incubation (Fig.3, IP c-FLIP), as
7 previously reported [39]. USP8 was constitutively present also in the HER3-ITCH complex,
8 and its expression slightly increased upon incubation with 9F7-F11 up to 1h (Fig.3, IP
9 HER3), as we previously demonstrated [33]. The strong reduction of USP8 level in the two
10 complexes at 2h suggests that after contributing to the formation of these complexes, USP8
11 leaves them to favor c-FLIP_L and HER3 downregulation. Altogether, these results
12 demonstrated that 9F7-F11 induced USP8 recruitment to stabilize ITCH, and then, the
13 USP8-ITCH complex binds to the ITCH targets c-FLIP_L and HER3, allowing their
14 ubiquitination and proteasomal degradation.

15

16 **ITCH and USP8 silencing by siRNA inhibits 9F7-F11-induced c-FLIP ubiquitination and** 17 **proteasomal degradation**

18 To confirm ITCH involvement in antibody-induced c-FLIP ubiquitination, we analyzed c-FLIP
19 ubiquitination after immunoprecipitation using an anti-c-FLIP antibody of protein extracts from
20 BxPC3 cells transfected with anti-*ITCH* (siITCH) or scramble control (siSC) siRNAs and pre-
21 treated with MG132 before incubation with 9F7-F11 and/or NRG1 for 4h (Fig.4a). We
22 observed c-FLIP_L poly-ubiquitination and ITCH co-immunoprecipitation in siSC cells
23 incubated with 9F7-F11 alone or with NRG1, but not in siITCH cells (Fig.4a). Moreover, in
24 siSC cells, 9F7-F11 reduced USP8 interaction with c-FLIP_L and promoted ITCH recruitment,
25 allowing c-FLIP_L ubiquitination (Fig.4a). Western blot analysis confirmed c-FLIP_L and c-FLIP_S
26 degradation in siSC cells upon incubation with 9F7-F11 alone (Fig.4b). This effect was
27 abrogated by siRNA-mediated silencing of *ITCH* (Fig.4b) and *USP8* (Fig.4c). These results

1 underlined ITCH and USP8 main role in 9F7-F11-induced c-FLIP degradation through
2 ubiquitination. Finally, ITCH downregulation in 9F7-F11-treated siUSP8, but not in siSC cells
3 (Fig.4c) suggests that USP8 deubiquitinates and stabilizes ITCH, which then promotes rapid
4 c-FLIP ubiquitination and proteosomal degradation [33]. In control cells (medium alone),
5 USP8 knock-down induced c-FLIP downregulation, as compared with siSC treated cells
6 (Fig.4c), confirming that USP8 directly deubiquitinates c-FLIP [39].

7

8 **ITCH silencing or chemical inhibition blocks 9F7-F11-induced apoptosis and c-FLIP** 9 **degradation**

10 To determine whether ITCH-induced c-FLIP_L degradation had a major role in anti-HER3
11 antibody-induced cancer cell apoptosis, we assessed PARP and caspase-8/3 cleavage by
12 western blotting in siITCH and siSC BxPC3 cells incubated with 9F7-F11 alone (Fig.5a) or
13 with NRG1 (Fig.5b) for 48h. In siITCH cells, ITCH expression was strongly downregulated,
14 and 9F7-F11-induced c-FLIP_L degradation inhibited, compared with siSC cells (Fig.5a). This
15 effect was stronger in siITCH cells co-incubated with NRG1 and 9F7-F11 (Fig.5b). Inhibition
16 of c-FLIP_L degradation repressed 9F7-F11-induced apoptosis, as indicated by the lower level
17 of cleaved PARP, p41/43 and p18 fragments (caspase-8 cleavage), and p17/19 fragments
18 (caspase-3 cleavage) in siITCH cells compared with siSC cells (Fig.5a). We confirmed the
19 requirement of ITCH-mediated c-FLIP_L proteasomal degradation for 9F7-F11-induced
20 apoptosis using chlorimipramine (CI), a specific ITCH chemical inhibitor that is known to
21 induce apoptosis at high dose, and irreversibly blocks ITCH by binding to its substrate pocket
22 [40]. In the absence of 9F7-F11 antibody, incubation with high dose of CI (30 μM) induced
23 apoptosis of MDA-MB-468 cells, as indicated by PARP and caspase-8/9/3 cleavage (Fig.5c),
24 as previously described [40,41]. However, pre-incubation with 15 μM CI for 48h completely
25 repressed 9F7-F11-induced caspase activation and apoptosis (Fig.5c), probably by inhibiting
26 ITCH binding to its substrate. When we combined higher dose (30μM) of CI with 9F7-F11,
27 we restored PARP cleavage induced by CI, albeit antibody-induced apoptosis is still

1 repressed. ITCH blockade by 15 μ M CI in 9F7-F11-treated cells was associated with the
2 disappearance of ITCH ubiquitination, and inhibition of antibody-induced c-FLIP_L and HER3
3 degradation, compared with cells incubated with medium or 9F7-F11 alone (Fig.5d). In the
4 agreement, ITCH chemical inhibitor CI reduced the percentage of apoptotic cells from 47.5%
5 to 21.5% in NRG1/9F7-F11-treated BxPC3 cells (Fig.5e), with a stronger effect on late
6 apoptosis; this reduction being also observed in CI-pre-incubated BxPC3 cells and further
7 treated with 9F7-F11 alone, in comparison with medium-pre-incubated antibody-treated cells
8 (Fig.5e). Similarly, *ITCH* RNA silencing of NRG1/9F7-F11-treated BxPC3 cells (siITCH)
9 decreased apoptosis to 29.4%, with regard to 47.8%-apoptosis observed in siSC cells
10 (Fig.5f). This reduction of apoptosis was also observed in siITCH BxPC3 cells treated with
11 9F7-F11 alone, compared with siSC cells (Fig.5f). As positive control, staurosporine induced
12 68.4%-cell apoptosis at 20h-post-treatment (Additional file 1: Figure S3). Taken together,
13 these results emphasized that the anti-HER3 antibody 9F7-F11 induces ITCH activation,
14 leading to HER3 and c-FLIP degradation to allow caspase-mediated apoptosis.

15

16 **9F7-F11 activates the extrinsic apoptotic pathway through FAS and DR5 upregulation,** 17 **TRAIL expression and DcR2 downregulation**

18 To test whether DRs were involved in 9F7-F11-induced apoptosis, we analyzed the
19 expression of factors involved in the extrinsic pathway by western blotting after incubation of
20 various cancer cell lines with 9F7-F11. Compared with untreated cells, the FAS and DR5
21 receptors were upregulated at 72h of incubation until the end of the experiment (120h)
22 (Fig.6a). TRAIL precursor (mTRAIL), which induces DR5 activation via a paracrine or
23 autocrine loop, was also overexpressed at 72h, except for DU145. The decoy receptor DcR2,
24 which inhibits DR5 activation by trapping TRAIL, was already downregulated at 24-48h
25 (Fig.6a). This demonstrated that 9F7-F11 induces the caspase-8-mediated extrinsic apoptotic
26 pathway by downregulating DcR2, leading to DR5 upregulation and TRAIL expression.

27 To test whether activation of the extrinsic apoptotic pathway induces DISC formation, we
28 immunoprecipitated BxPC3 cells with an anti-DR5 antibody at different time points during

1 incubation with 9F7-F11 (Fig.6b). In untreated cells (i.e., without caspase-8 activation to form
2 a pre-DISC), FADD and pro-caspase 8 were weakly immunoprecipitated with DR5 compared
3 to IgG baseline. Conversely, 9F7-F11 promoted p41/43 and p18 (caspase-8 cleavage) and
4 FADD recruitment, indicating DR5-DISC formation. Interestingly, FADD activation, as well as
5 p41/43 caspase 8 cleavage, seem to fluctuate with lower levels observed at 48h and 96h,
6 and overexpression at 24h, 72h and 120h-post 9F7-F11 treatment. Strong global activation
7 was observed at 120h-end of the experiment (Fig.6b), thus correlating DR5/FADD
8 overexpression with increased caspase-8 activation to induce the extrinsic apoptotic
9 pathway.

10

11 **9F7-F11 activates also the intrinsic mitochondrial apoptotic pathway**

12 9F7-F11 induced caspase-8 activation followed by caspase-9/3 cleavage (Fig.1). Analysis by
13 western blotting of caspase-mediated apoptosis in BxPC3 cells incubated with 9F7-F11
14 showed that the anti-HER3 antibody also induced BID cleavage and formation of the
15 truncated form tBID from 72h-96h until the end of the experiment (120h) (Fig.7a), suggesting
16 that caspase-8 activation induces BID cleavage to favor the mitochondrial death pathway.
17 9F7-F11 also upregulated BAX and promoted dimer formation at 72h (Fig.7a), strengthening
18 the involvement of mitochondria to promote complete activation of caspase-3 via caspase-9.
19 The induction of the p53 transcriptional target BIM (at 48h; Fig.7a) allows mitochondria
20 amplification of caspase-3 activation. These effects were confirmed also in MDA-MB-468
21 cells incubated with 9F7-F11 in a slight different time-frame (Fig.7b). We only observed 9F7-
22 F11-induced BIM induction in BAX-deficient DU145 cells [42] (Fig.7b). These results
23 demonstrated the involvement of the intrinsic apoptotic pathway (BID truncation, BAX
24 upregulation and BIM expression to induce full caspase-9 activation) in cancer cells
25 incubated with 9F7-F11.

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28

1 **9F7-F11 induces downregulation of the pro-survival proteins c-IAP2 and XIAP**

2 9F7-F11 sensitized tumor cells to caspase-mediated apoptosis through ITCH-dependent c-
3 FLIP downregulation. Pro-survival proteins from the IAP family inhibit apoptosis [43]. For
4 efficient apoptosis inhibition, XIAP is stabilized by interaction with the deubiquitinase USP9X
5 and then promotes caspase-3 degradation through ubiquitination [44]. We previously
6 demonstrated that anti-HER3 antibodies inhibit XIAP phosphorylation to favor apoptosis [2].
7 Here, western blotting analysis (Fig.8) showed that in 9F7-F11-treated BxPC3 cells, cIAP2
8 and XIAP expression was reduced starting at 48h-72h and completely downregulated at 96h.
9 The progressive USP9X downregulation, which occurred concomitantly with that of cIAP2
10 and XIAP, could be responsible for XIAP degradation. We obtained similar results with other
11 cancer cell lines (MDA-MB-468 and DU145 cells), but with slower kinetics (Fig.8). This
12 indicated that 9F7-F11 induces USP9X downregulation leading to XIAP degradation, which
13 allows DR5-mediated caspase-dependent apoptosis.

1 Discussion

2 Here, we report that ITCH-dependent proteasomal degradation of c-FLIP induced by the anti-
3 HER3 antibody 9F7-F11 favors DR5/caspase 8-mediated apoptosis of tumor cells. This
4 mechanism (as illustrated in Figure 9) can explain how anti-HER3 antibodies directly induce
5 cancer cell apoptosis [2,45,46] and counteract the HER3-mediated apoptosis inhibition
6 observed in cancer cells resistant to chemotherapy [3,4]. HER3 silencing [1,47] or
7 pharmacological inhibition [2] directly restores tumor-specific apoptosis, underlying its critical
8 role in cell death inhibition. HER3 degradation induced by specific antibodies [2,33,48,49] is
9 a marker of pre-clinical drug efficacy [2,32,45,46], and is frequently associated with cancer
10 cell apoptosis induction [2,45,46]. We previously demonstrated that in cancer cells, 9F7-F11
11 blocks the PI3K/AKT pathway [2,32,33], induces HER3 downregulation and promotes cell
12 apoptosis [2,33], leading to *in vivo* tumor regression [2,32]. The binding to HER3 and
13 biological effects on tumor cells of 9F7-F11 are paradoxically facilitated by the natural ligand
14 NRG1 [32]. By hijacking NRG1 addiction of cancer cells to promote its inhibitory effects on
15 NRG1-mediated tumor growth and resistance, the allosteric non NRG1-competing 9F7-F11
16 displays a unique potential for targeted treatment of NRG1-positive cancers [32].
17 HER3 ubiquitination and degradation induced by 9F7-F11 mainly occur through JNK1/2-
18 dependent ITCH activation, and are regulated by the deubiquitinases USP8 and USP9X
19 [33]. Here, we showed that upon incubation of cancer cells with 9F7-F11, the E3 ubiquitin
20 ligase ITCH interacts with c-FLIP to trigger c-FLIP ubiquitination and degradation,
21 concomitantly with early ITCH recruitment to HER3 [33]. In this setting, 9F7-F11 induces
22 JNK1/2 activation to phosphorylate ITCH on Thr222. Other studies identified JNK1/2 as the
23 main regulator of ITCH-induced c-FLIP degradation after TNF α stimulation [26] or AKT
24 inhibition in glioblastoma [27]. c-FLIP degradation via JNK/ITCH activation has been recently
25 described to sensitize tamoxifen-resistant breast cancer to TRAIL-induced cell death [28].
26 9F7-F11 could be useful to bypass resistance to chemotherapy in breast cancer by favoring

1 c-FLIP degradation via JNK/ITCH activation. In basal conditions (medium alone), the
2 deubiquitinase USP8 contributes to stabilization of c-FLIP, as shown here (Fig.3 and 4) and
3 by others [39], and of ITCH [27,33]. Conversely, during incubation with 9F7-F11, USP8
4 leaves the ITCH-c-FLIP complex, allowing JNK1/2-mediated ITCH activation for c-FLIP
5 ubiquitination. ITCH and USP8 silencing experiments highlighted the role of this
6 ubiquitination/deubiquitination process in modulating 9F7-F11-induced c-FLIP and HER3
7 degradation, and also in the inhibition of caspase-8-mediated apoptosis of tumor cells. This
8 links c-FLIP downregulation by 9F7-F11 with antibody-induced caspase-8 activation. In
9 addition, antibody treatment disrupted the basal USP8-HER3 interaction to favor ITCH-
10 mediated HER3 ubiquitination and proteasomal degradation. In conclusion, the
11 deubiquitinase USP8 acts by co-regulating c-FLIP (Fig.4), ITCH and HER3 [33] stability, and
12 this triple regulation is affected by 9F7-F11. Interestingly, it has been reported that a
13 synthetic USP8 inhibitor also induces downregulation of receptor tyrosine kinases, including
14 HER3 and c-MET, leading to inhibition of cell survival/proliferation and tumor regression in
15 mice xenografted with gefitinib-resistant non-small cell lung cancer cells [50]. Similarly to
16 9F7-F11 activity, vitamin E analogues (α -tocopherol derivatives) inhibit the HER3-mediated
17 AKT pro-survival pathway and the anti-apoptosis factors c-FLIP and survivin to favor
18 caspase-mediated apoptosis in cisplatin-resistant ovarian cancer [51]. Targeting c-FLIP has
19 been also proposed in various cancers, mainly indirectly by using chemotherapies such as
20 cisplatin, 5-fluorouracil, gemcitabine, etoposide and paclitaxel [14].

21 We demonstrated that 9F7-F11 induces DR5 upregulation and TRAIL expression, leading to
22 DR5-mediated caspase-8 activation through the formation of the DR5-DISC complex.
23 Chemical compounds, such as dibenzylideneacetone, also upregulate DR5, via activation of
24 ROS-mediated C/EBP homologous transcription factor (CHOP) that induces transcription of
25 pro-apoptotic proteins [52]. CHOP-dependent DR5 upregulation and ROS production
26 promote TRAIL-induced apoptosis through downregulation of XIAP, survivin, and c-FLIP_L
27 and c-FLIP_S [52]. Similarly, the natural molecule zerumbone, a sesquiterpene from tropical

1 ginger, induces DR5 upregulation via ROS-mediated activation of the MAP kinases ERK1/2
2 and p38, leading to DR5/TRAIL-mediated apoptosis [53]. Oxidative stress, through ROS
3 production, is often associated with apoptosis via JNK1/2 activation. Some therapeutic
4 antibodies promote apoptosis together with ROS accumulation, leading to JNK activation
5 [54,55]. Anti-HER3 antibodies, such as MM-121 and hMP-RM-1 that downregulate survivin
6 [4], or 9F7-F11 that inhibits XIAP phosphorylation [2], favor apoptosis and reduce cell
7 survival and proliferation. Here, we showed that 9F7-F11 represses the expression of the
8 survival proteins XIAP and c-IAP2 and also of c-FLIP, concomitantly with DR5 upregulation
9 and TRAIL expression induction. This suggests that antibody-induced caspase-8-mediated
10 apoptosis involves DR5 activation by TRAIL autocrine loop or by ROS-dependent DR5
11 aggregation for DISC formation. It is worth noting that in our setting, DR5-DISC formation
12 started early with the recruitment of FADD and pro-caspase 8, before DR5 upregulation.
13 Therefore, anti-HER3 antibody-triggered DR5 aggregation and DISC formation might occur
14 independently of TRAIL induction to activate caspase-8-mediated apoptosis. Our findings
15 showed that 9F7-F11-induced apoptosis involves also the mitochondrial pathway with the
16 induction of BID cleavage by caspase-8, BAX upregulation and BIM expression induction,
17 leading to mitochondria-dependent caspase-9 activation and full caspase-3 activation. This is
18 in agreement with previous work showing that siRNA-mediated HER3 downregulation
19 induces BAX-BAK-mediated apoptosis [47]. Upon incubation with 9F7-F11, we observed BID
20 cleavage and BIM expression also in DU145 prostate cancer cells, which are BAX-deficient
21 [42] but express BAK that could replace BAX to induce caspase-9 activation. It has been
22 demonstrated that BAK is preferentially activated by tBID and BAX by BIM, to modulate the
23 response to chemotherapy [56]. Indeed, anti-HER3 antibody-induced apoptosis in DU145
24 cells was lower than in the other tested cancer cell lines, probably because BAX deficiency
25 prevented BIM-mediated apoptosis.

26

27

1 **Conclusions**

2 We provide evidence that the allosteric non-NRG1 competing modulator 9F7-F11, sensitizes
3 tumor cells to caspase-mediated apoptosis through ITCH-dependent degradation of c-FLIP,
4 and independently of ligand addiction. The description of the multiple modes of action of the
5 anti-HER3 antibody 9F7-F11 not only adds to our basic understanding of dysregulated
6 signaling in cancer, but might help the selection of drug combinations and clinical indications
7 for 9F7-F11 in NRG1-addicted or NRG1-rearranged cancer.

1 **Declarations**

2 **Abbreviations**

3 7-AAD: A-Aminoactinomycin D; AIP: BAD: Bcl-2-associated death promoter; BID: BH3
4 interacting-domain death agonist; BIM: Bcl-2-like protein 11; cIAP2: Cellular inhibitor of
5 apoptosis protein 2; CI: Chlorimipramine; DED: Death effector domain; DISC: Death-inducing
6 signaling complex; DR: Death receptor; FADD: Fas-associated protein with death domain; c-
7 FLIP: FLICE-like inhibitory protein; ITCH: E3 ubiquitin-protein ligase Itchy homolog; JNK: c-
8 Jun N-terminal kinase; NRG1: Neuregulin 1; PARP: poly(ADP-ribose) polymerase; SD:
9 Standard deviation; USP8: Ubiquitin-specific peptidase 8; XIAP: X-linked inhibitor of
10 apoptosis protein;

11

12 **Ethics approval and consent to participate**

13 Not applicable

14

15 **Consent for publication**

16 Not applicable

17

18 **Availability of data and material**

19 All data generated or analysed during this study are included in this published article (and its
20 supplementary information files)

21

22 **Competing interests**

23 OD, JMB, and JFP are employed by GamaMabs Pharma. AP, CL and TC are inventors in
24 the WO2012/156532 patent “Anti-human HER3 and uses thereof”, the WO2015/067986
25 patent “Neuregulin allosteric anti-HER3 antibody”, and the WO2016/177664 patent “Low-
26 fucose anti-human HER3 antibodies and uses thereof”. OD, JMB and JFP are inventors in

1 the WO2016/177664 patent “Low-fucose anti-human HER3 antibodies and uses thereof”.
2 The other authors declare no conflict of interest.

3

4 **Funding**

5 This work was supported by the program “Investissement d’Avenir” (grant agreement:
6 LabexMabImprove, ANR-10-LABX-53-01) and by the grant AAP13 “Fonds Unique
7 Interministeriel” FUI UmAbHER3 F120402M. CLC was supported by INSERM through the
8 FUI UmAbHER3. CS was supported by the Conseil Régional de Bourgogne, the European
9 Union and the University of Burgundy.

10

11 **Authors’ Contributions**

12 CLC and TC contributed to the conception and design of the study. CLC, YL, OD, RL, and
13 TC contributed to the development of the methodology. CLC and TC acquired, analyzed and
14 interpreted the data. CLC, CS, CL, MAP, AP and TC wrote the manuscript. JMB, JFP, AP
15 and TC edited and supervised the manuscript, and provided critical comments. All authors
16 read and approved the final manuscript.

17

18 **Acknowledgements**

19 We thank G. Heintz and S. Bousquié for excellent technical assistance.

20

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6

1 **Figure legends**

2

3 **Figure 1. 9F7-F11-induced tumor cell apoptosis occurs through caspase-8/9/3**
4 **activation and PARP cleavage.** BxPC3, MDA-MB-468 or DU145 cancer cells were
5 incubated with the anti-HER3 antibody 9F7-F11. Caspase-8, -9 and -3 and PARP cleavage
6 were analyzed by western blotting and cell lysates prepared at different time points during
7 antibody incubation. Quantification of signal intensity (SI) with ImageJ software is indicated
8 below the images (relative to untreated control measured as 1.0 ± 0). Significant increase or
9 decrease of the densitometry, compared to control, is indicated in bold. M: medium alone;
10 p41/43 and p18 C8c: caspase-8 cleavage; p37 C9c: caspase-9 cleavage; p17/19 C3c:
11 caspase-3 cleavage; PARPc: PARP cleavage. β -tubulin was evaluated as loading control.

12

13 **Figure 2. 9F7-F11 induces c-FLIP downregulation by proteasomal degradation. (a)**
14 Cancer cells were incubated with NRG1 or 9F7-F11. After cell lysis at different time points, c-
15 FLIP_L, USP8 and USP9X expression, as well as ITCH expression and phosphorylation (p)
16 were analyzed by western blotting. **(b)** BxPC3 cells were incubated with 9F7-F11 for 3h.
17 HER3, c-FLIP_L and c-FLIP_S expression were analyzed by western blotting. **(c)** BxPC3 and
18 MDA-MB-468 cells were incubated with NRG1 or 9F7-F11 for 48h or 96h, before detection of
19 HER3 and c-FLIP_L expression by western blotting. **(d)** After pre-incubation or not with 10 μ M
20 MG132 for 4h, BxPC3 cells were incubated with 9F7-F11 with or without NRG1. After cell
21 lysis, expression of HER3 and c-FLIP_L was assessed in whole cell lysates by western
22 blotting. The rabbit anti-HER3 polyclonal antibody C-17 (Santa Cruz Biotechnology) was
23 used for detection. Protein level was measured with the ImageJ software and indicated as
24 signal intensity (SI), relative to untreated control ($SI=1.0 \pm 0$). Significant increase or decrease
25 of the densitometry, compared to control, is indicated in bold. β -tubulin was evaluated as
26 loading control.

27

1 **Figure 3. USP8-regulated ITCH interaction with c-FLIP mediates 9F7-F11-induced c-**
2 **FLIP ubiquitination.** BxPC3 cells were incubated with NRG1 or/and 9F7-F11 for various
3 times. After cell lysis in CHAPS buffer, 2 mg of total protein extracts were co-
4 immunoprecipitated with the anti-HER3 antibody 2F12 (Millipore) against HER3 C-terminal
5 tail. Then, the first soluble supernatant was co-immunoprecipitated with the rabbit anti-c-FLIP
6 polyclonal antibody H-202 (Santa Cruz Biotechnology) that targets both c-FLIP_L and c-FLIP_S.
7 The presence of ITCH and USP8 in the two immunoprecipitates was assessed by western
8 blotting. HER3 and c-FLIP ubiquitination status were assessed using the anti-K48 ubiquitin
9 antibody. Whole cell lysates (WCL) were analyzed using the appropriate antibodies.
10 Quantification of signal intensity (SI) with ImageJ software is indicated below the images, in
11 comparison to SI=1.0±.0 for untreated control. Significant increase or decrease of the
12 densitometry, compared to control, is indicated in bold. β-actin was evaluated as loading
13 control.

14
15 **Figure 4. ITCH or USP8 silencing by siRNA inhibits 9F7-F11-induced c-FLIP**
16 **ubiquitination and proteasomal degradation.** (a) BxPC3 cells were transfected with 50 nM
17 ITCH-specific siRNA (siITCH) or with control scramble siRNA (siSC) for 72h, before pre-
18 treatment with 10 μM MG132 for 4h. Cells were then incubated with 9F7-F11, with or without
19 NRG1, or medium as control for 4h. After immunoprecipitation of total protein extracts (2 mg)
20 with the anti-c-FLIP antibody H-202, c-FLIP, ITCH and USP8 expression and c-FLIP
21 ubiquitination were analyzed by western blotting. BxPC3 cells were transfected with siSC,
22 siITCH (b) or siUSP8 (c) for 72h, and then incubated with 9F7-F11 for 4h. Expression of
23 ITCH, c-FLIP and USP8 was assessed in total protein extracts by western blotting. Protein
24 level was measured with the ImageJ software and indicated as signal intensity (SI), relative
25 to untreated control (SI=1.0±.0). Significant increase or decrease of the densitometry,
26 compared to control, is indicated in bold. β-tubulin was evaluated as loading control.

27

1 **Figure 5. ITCH silencing or chemical inhibition blocks 9F7-F11-induced apoptosis and**
2 **c-FLIP degradation.** siSC- and siITCH-transfected BxPC3 cells were incubated with 9F7-
3 F11 alone (a), or with NRG1 (b) for 48h before detection by western blotting of ITCH and c-
4 FLIP_L expression, and PARP/caspase cleavage. MDA-MB-468 cells were incubated with
5 increasing doses of chlorimipramine (CI) for 24h (c), or with 15 μM CI (d) before incubation
6 with 9F7-F11 for 24h. Total protein extracts were analyzed by western blotting to evaluate
7 PARP and caspase cleavage (c), ITCH ubiquitination and expression and c-FLIP and HER3
8 expression (d). Quantification of signal intensity (SI) with ImageJ software is indicated below
9 the images. No protein expression was measured as 0.0±0. Significant decrease or increase
10 of the densitometry, compared to control, is indicated in bold. BxPC3 cells were left
11 untreated or pre-incubated with ITCH chemical inhibitor CI for 48h before treatment with 9F7-
12 F11 with or without NRG1. Apoptosis was measured at 96h by flow cytometry after cell
13 labelling with Annexin V/7-AAD (e). siSC and siITCH-transfected BxPC3 cells were treated
14 with 9F7-F11 alone or with NRG1. Western blot was performed to check ITCH reduction in
15 siITCH-transfected BxPC3 cells. Apoptosis was measured at 96h by flow cytometry after cell
16 labelling with Annexin V/7-AAD (f). ***P*<0.01, ****P*<0.001, ns not significant.

17

18 **Figure 6. 9F7-F11 activates the extrinsic apoptotic pathway through FAS and DR5**
19 **upregulation, TRAIL expression induction and DcR2 downregulation.** (a) BxPC3, MDA-
20 MB-468, and DU145 cells were treated with 9F7-F11 for various times. FAS, DR5, mTRAIL
21 and DcR2 expression were detected by western blotting. (b) BxPC3 cells were incubated
22 with 9F7-F11. After cells lysis at different time points, total protein extracts (2 mg) were co-
23 immunoprecipitated with the human anti-DR5 polyclonal antibody D4E7 (Cell Signaling
24 Technology) or with human anti-IgG as control. The presence of DISC components
25 (caspase-8, FADD and DR5) was assessed in immunoprecipitates and whole cell lysates
26 (WCL) by western blotting. Protein level was measured with the ImageJ software and
27 indicated as signal intensity (SI), relative to untreated control (SI=1.0±0). Significant increase

1 or decrease of the densitometry, compared to control, is indicated in bold. β -tubulin was
2 evaluated as loading control.

3

4 **Figure 7. 9F7-F11 activates the intrinsic mitochondrial apoptosis pathway by inducing**
5 **caspace-8-mediated BID cleavage, BAX activation and BIM expression.** BxPC3 (a),
6 MDA-MB-468 and DU145 (b) cells were incubated with 9F7-F11 for various times. BID
7 cleavage (tBID), BAX activation and BIM expression were analyzed by western blotting.
8 Quantification of signal intensity (SI) with ImageJ software is indicated below the images. No
9 protein expression was measured as 0.0 ± 0 . Significant decrease or increase of the
10 densitometry, compared to untreated control, is indicated in bold. β -tubulin was evaluated as
11 loading control.

12

13 **Figure 8. 9F7-F11 induces downregulation of the pro-survival proteins c-IAP2 and**
14 **XIAP.** Cells were incubated with 9F7-F11, before western blot analysis of cIAP2 and XIAP
15 (survival factors) and of UP9X (deubiquitinase) expression in protein lysates prepared at
16 different time points. Quantification of signal intensity (SI) with ImageJ software is indicated
17 below the images (relative to untreated control measured as 1.0 ± 0). Significant increase or
18 decrease of the densitometry, compared to control, is indicated in bold. β -tubulin was
19 evaluated as loading control.

20

21 **Figure 9.** The representation illustrates a proposed model for ITCH-dependent proteasomal
22 degradation of c-FLIP induced by 9F7-F11 to promote DR5/caspase-8 apoptosis. See text
23 for details.

24

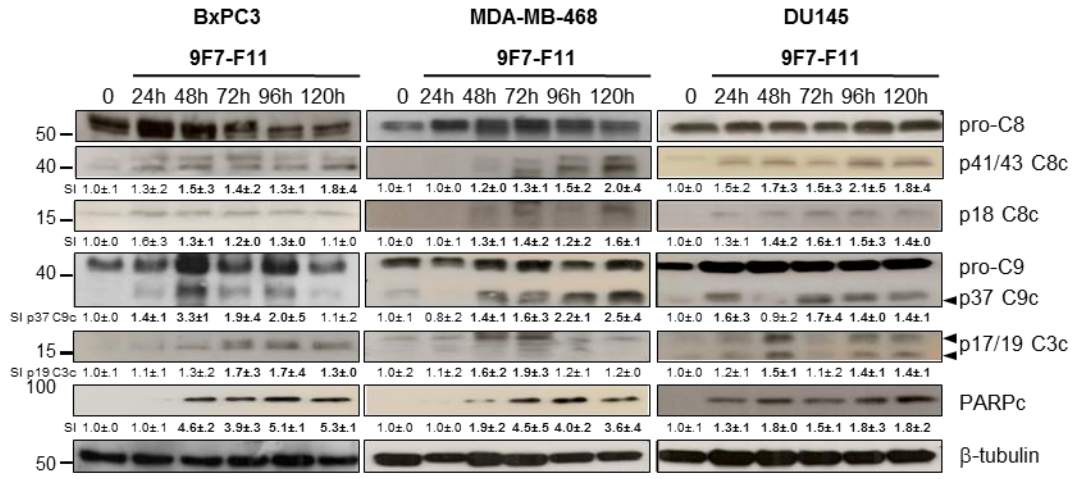
1 **Additional file 1 :**

2 **Figure S1.** (a) Flow cytometry analysis of EGFR, HER2 and HER3 expression at the
3 membrane of BxPC3, DU-145 and MDA-MB-468 cells. (b) Western blot analysis of receptor
4 and NRG1 expression, and USP8, USP9 and ITCH expression in whole lysates of BxPC3,
5 DU-145 and MDA-MB-468 cell lines.

6 **Figure S2.** 9F7-F11-induced apoptosis of tumor cells occurs through activation of caspase-
7 8/9/3 and PARP cleavage. BxPC3 cells were incubated with the anti-HER3 antibody 9F7-F11
8 and/or NRG1 for 60h. Total proteins extracts were analyzed by western blotting for caspase-
9 8, -9 and -3, and PARP cleavage. Quantification of signal intensity (SI) with ImageJ software
10 is indicated below the images (relative to untreated control measured as 1.0 ± 0). Significant
11 increase or decrease of the densitometry, compared to control, is indicated in bold.

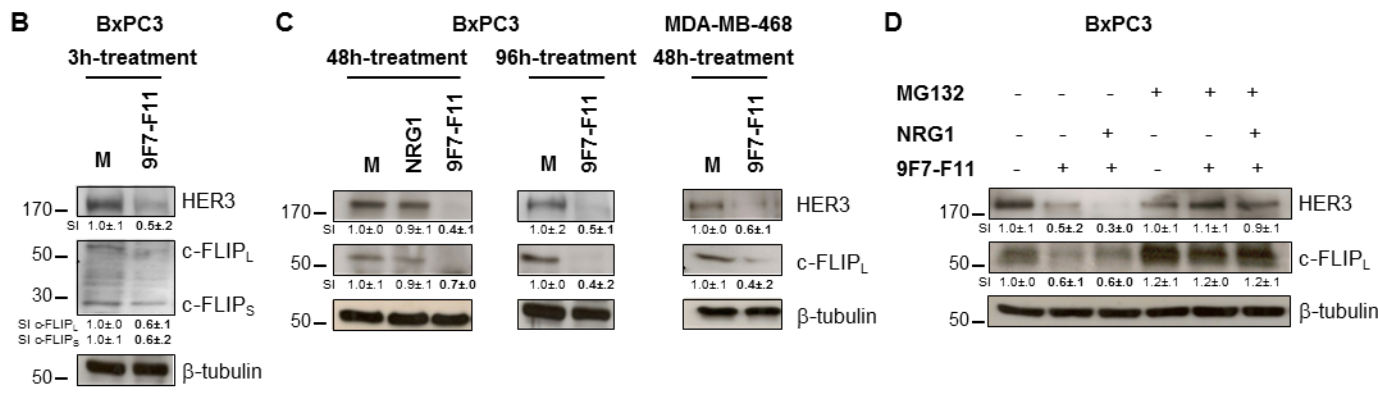
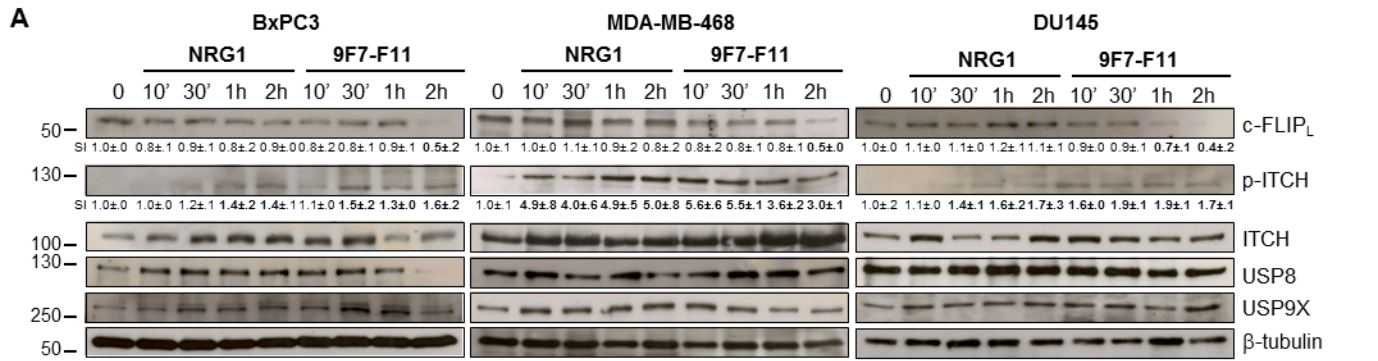
12 **Figure S3.** BxPC3 cells were treated with 300nM Staurosporine, as apoptosis positive
13 control. Apoptosis was measured at 6h and 20h by flow cytometry after cell labelling with
14 Annexin V/7-AAD.

Fig. 1



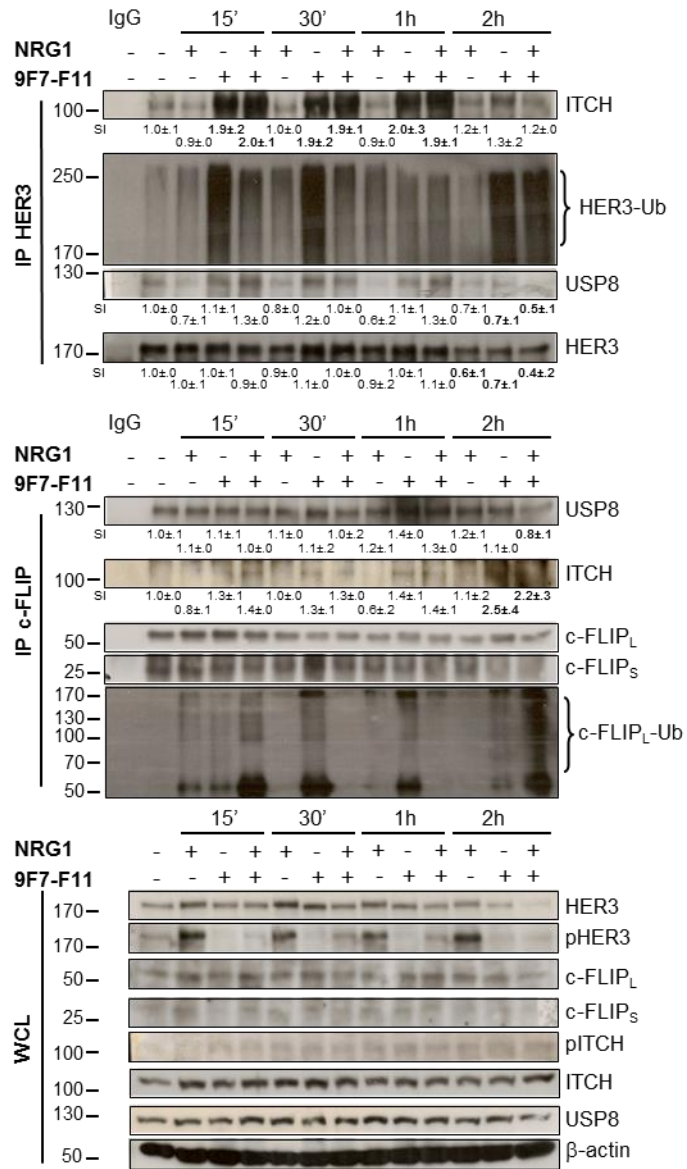
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Fig. 2



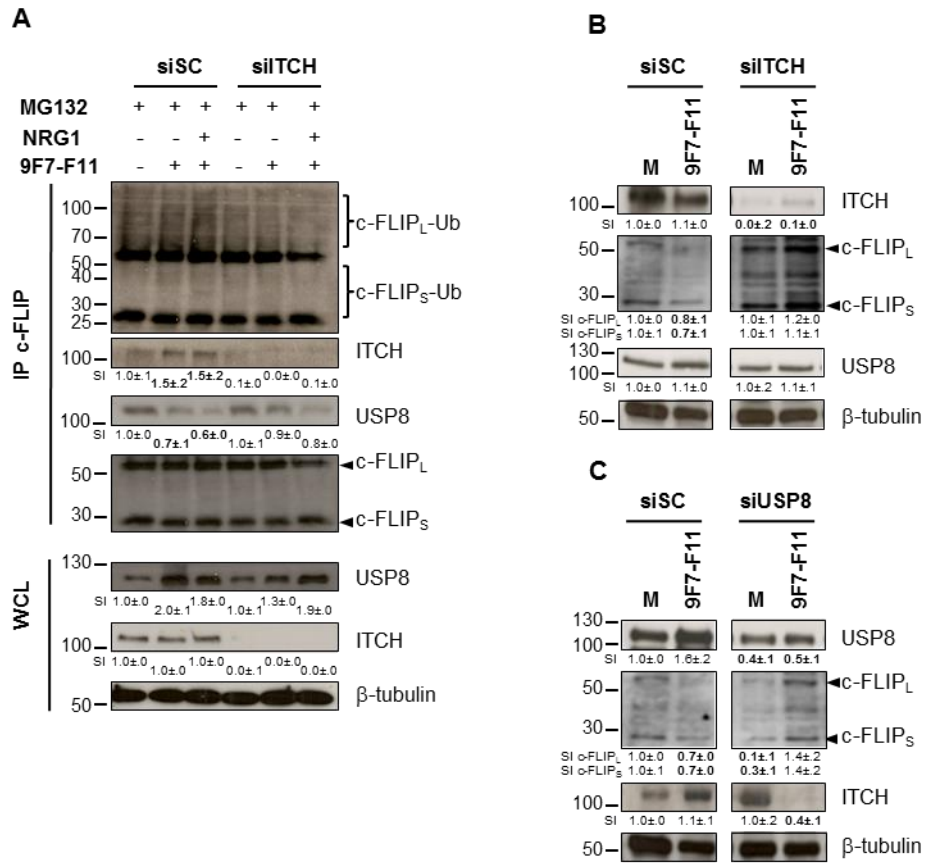
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Fig. 3



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Fig. 4



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Fig. 5

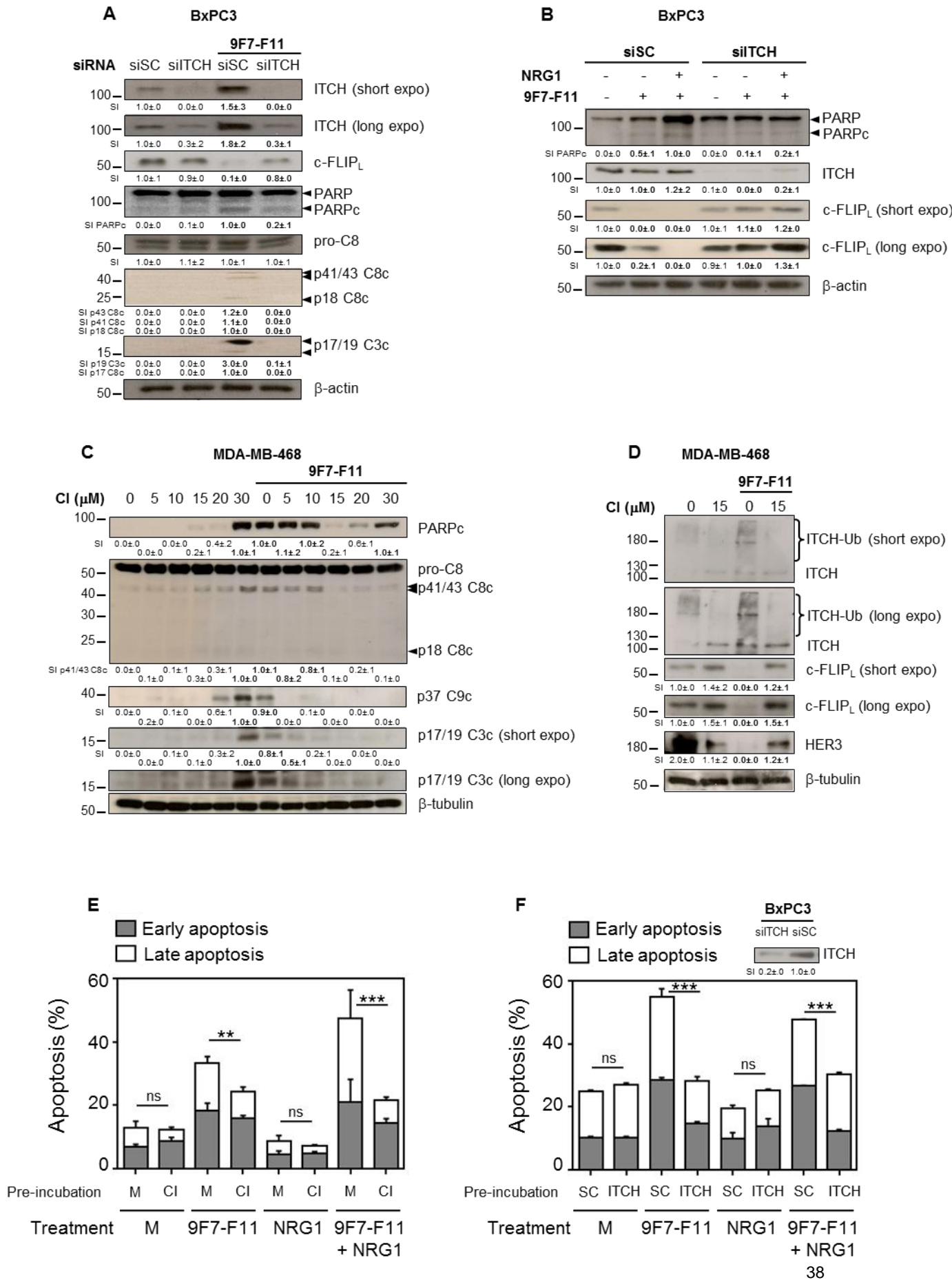


Fig. 6

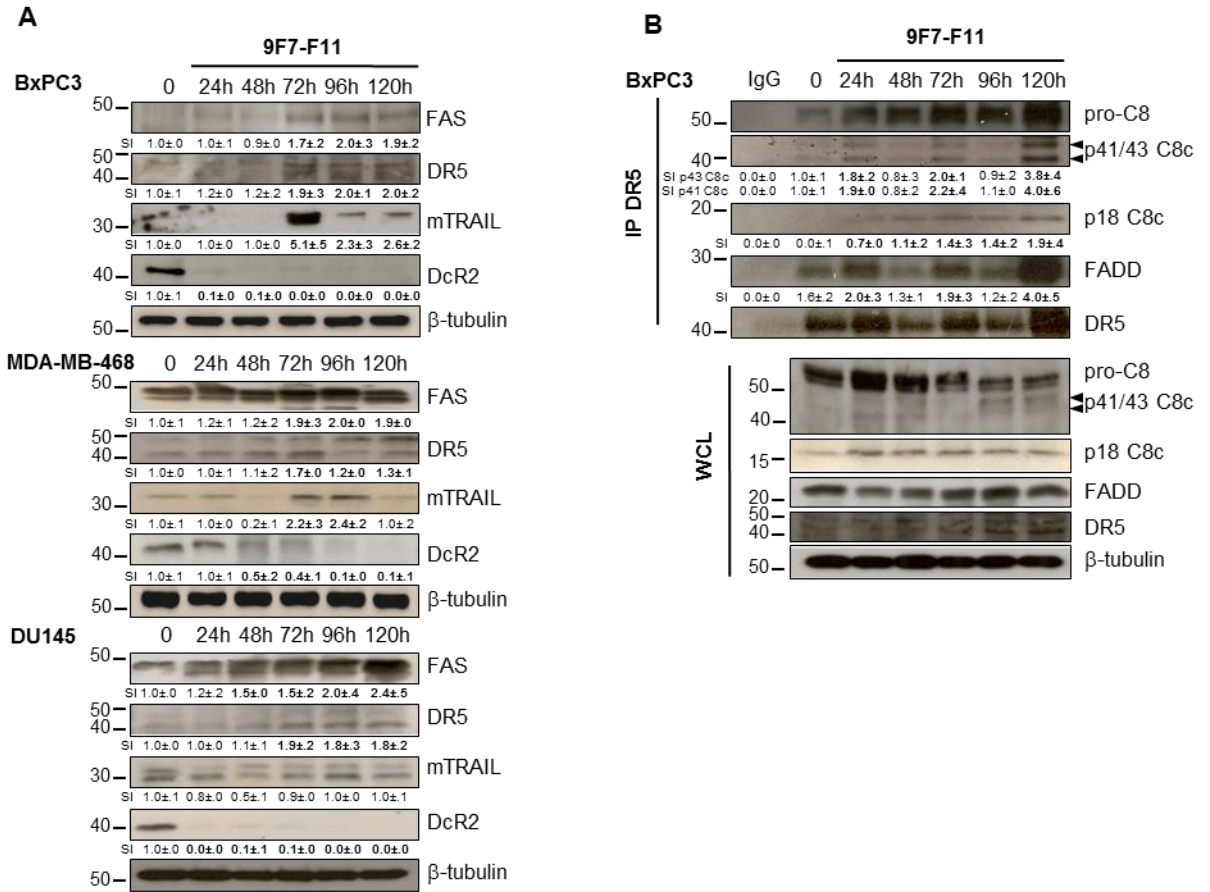
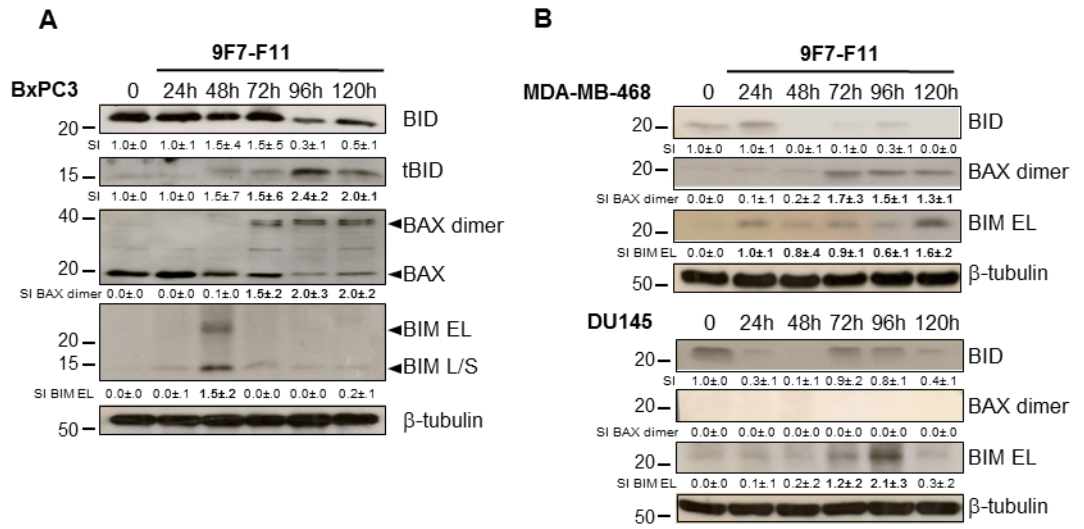
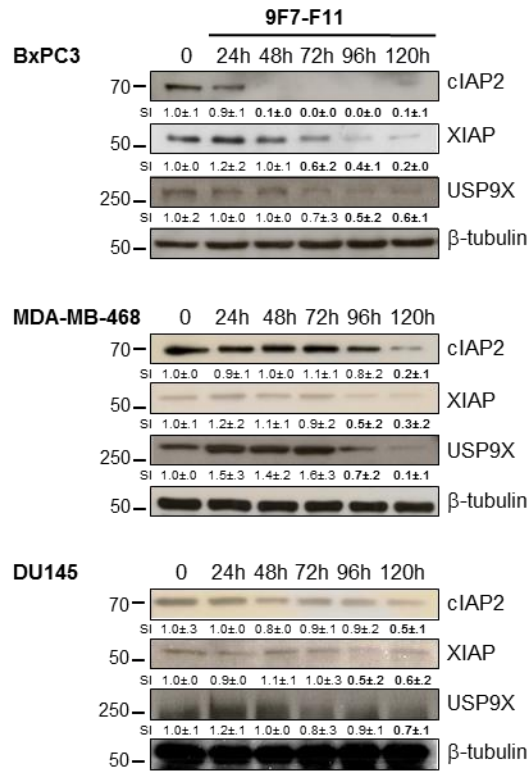


Fig. 7



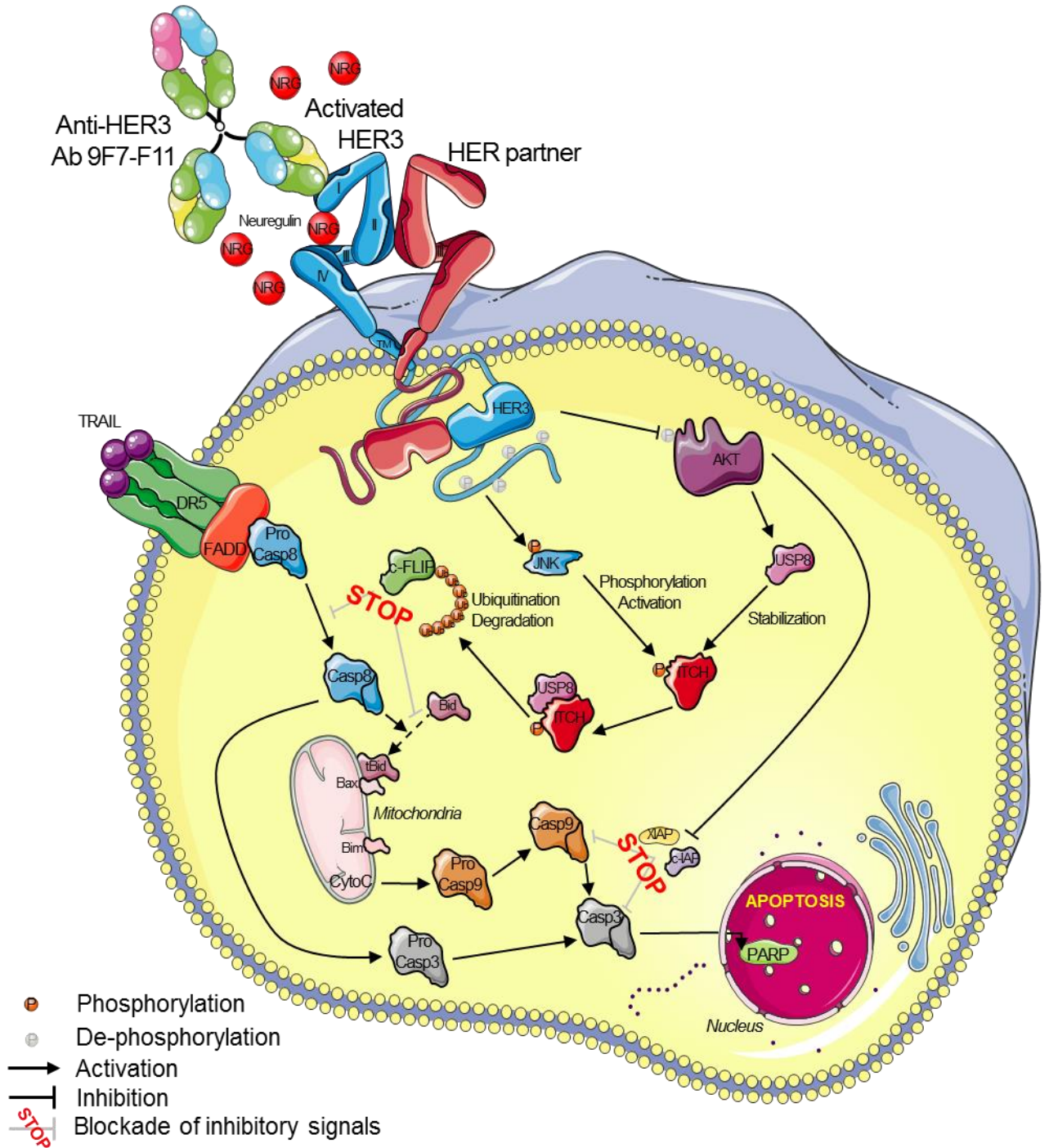
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Fig. 8



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Fig. 9



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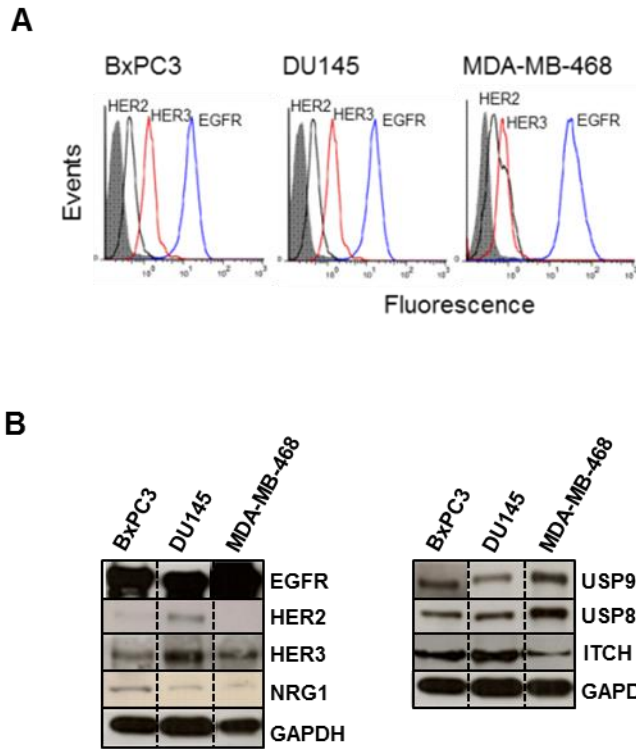


Figure S1. (a) Flow cytometry analysis of EGFR, HER2 and HER3 expression at the membrane of BxPC3, DU145 and MDA-MB-468 cells. (b) Western blot analysis of receptor and NRG1 expression, and USP8, USP9 and ITCH expression in whole cell lysates of BxPC3, DU145 and MDA-MB-468 cell lines.

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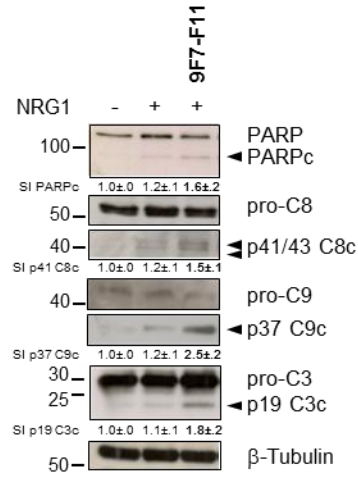


Figure S2. 9F7-F11-induced apoptosis of tumor cells occurs through activation of caspase-8/9/3 and PARP cleavage. BxPC3 cells were incubated with the anti-HER3 antibody 9F7-F11, and/or NRG1 for 60h. Total proteins extracts were analyzed by western blotting for caspase-8, -9 and -3, and PARP cleavage. Quantification of signal intensity (SI) with ImageJ software is indicated below the images (relative to untreated control measured as 1.0±.0). Significant increase or decrease of the densitometry, compared to control, is indicated in bold.

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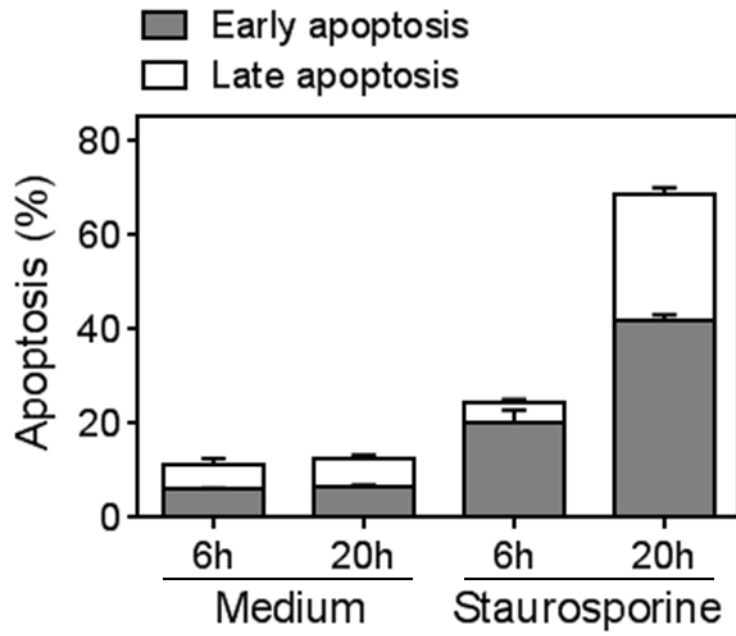


Figure S3. BxPC3 cells were treated with 300nM Staurosporine, as apoptosis positive control. Apoptosis was measured at 6h and 20h by flow cytometry after cell labelling with Annexin V/7-AAD.

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