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Short Report

Fragment N2, a caspase-3 generated RasGAP fragment, inhibits breast cancer metastatic progression

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WHAT's NEW?

RasGAP family members are negative modulators of Ras, an oncogene expressed by many tumors. The p120 RasGAP protein was the first identified RasGAP family member. Beyond regulation of Ras, p120 RasGAP triggers various signaling cascades and, as a caspase-3 substrate, plays important roles in stress sensing. Here we report that fragment N2, a p120 RasGAP fragment generated by caspase-3, prevents metastatic progression. This indicates that stress, via activation of caspase-3, can generate metastasis suppressor responses.

ABSTRACT

The p120 RasGAP protein negatively regulates Ras via its GAP domain. RasGAP carries several other domains that modulate various signaling molecules such as Rho. RasGAP is also a caspase-3 substrate. One of the caspase-3-generated RasGAP fragments, corresponding to amino acids 158-455 and called fragment N2, was previously reported to specifically sensitize cancer cells to death induced by various anticancer agents. Here we show that fragment N2 inhibits migration *in vitro* and that it impairs metastatic progression of breast cancer to the lung. Hence, stress-activated caspase-3 might contribute to the suppression of metastasis through the generation of fragment N2. These results indicate that the activity borne by fragment N2 has a potential therapeutic relevance to counteract the metastatic process.

INTRODUCTION

Metastasis is responsible for most cancer-related deaths[1] but preventing or inhibiting metastasis formation remains a challenge. Understanding the molecular mechanisms involved in the metastatic cascade is therefore crucial to develop therapeutical anti-metastatic drugs. Metastatic progression is a complex multistep process that includes the escape of cancer cells from the primary tumor, the intravasation into the lymphatic or hematogenous systems, the extravasation into the parenchyma of new distant sites and the colonization of these sites.[2] These steps are associated with increased motility, invasiveness, cell-cell binding modulation, and decreased adhesion of cells to their substratum.[2] Targeting specifically the molecular pathways that affect cell adhesion and migration represents prime anti-cancer strategies.

Among the most commonly deregulated signaling proteins in cancer are the Ras protein.[3] These are activated by guanine nucleotide exchange factors (GEFs) and negatively modulated by GTPase-activating proteins (GAPs).[4] There are ten different GAPs that regulate Ras[5] including DAB2IP and Rasal2 that have recently been found to be dual tumor and metastasis suppressors.[6,7] Whether other RasGAPs act as metastasis suppressors remains unknown.

p120 RasGAP (from now on referred to as RasGAP) is more than a mere negative modulator of the Ras pathway via its GAP domain.[8] Indeed its N-terminal moiety contains multiple domains, including SH2 and SH3 domains, that positively modulate kinases such as Cdk1.[9] The N-terminal region of RasGAP, in contrast to its C-terminal end, can lead to Ras activation.[10] This can explain for example why there is a weaker basal Ras activity in cells lacking RasGAP compared to wild-type cells.[11] RasGAP can therefore negatively or positively control Ras activation in a manner that is probably cell- and stimulus-dependent. RasGAP is also a caspase-3 substrate. The RasGAP/caspase-3 pair forms a stress-sensing module that induces survival signals in homeostasis perturbing conditions and apoptosis in the presence of excessive stress.[12] Stress sensing by this module relies on differential cleavage of RasGAP at low and high caspase-3 activity. In the presence of a low stress, caspase-3 cleaves RasGAP once, generating an amino-terminal fragment, called fragment N, that efficiently promotes cell survival in Ras/PI3K/Akt-dependent manner.[10,13,14] When the stress reaches unsustainable levels, caspase-3 further cleaves fragment N into two

smaller fragments, called N1 and N2, that no longer have the ability of stimulating Akt.[15] This terminates the Akt-dependent protecting signals, thereby favoring cell death.

Fragment N2 favors pro-apoptotic signaling, in cancer cells but not in non-malignant cells, in response to various anti-cancer agents.[16] Recently, fragment N2 was found to increase the adhesive capacity of cells.[17] This activity is carried by a ten amino acid sequence within fragment N2 that correspond to amino acid 317-326 of RasGAP.[17] These findings prompted us to test the ability of fragment N2 to inhibit metastatic progression *in vivo*. Here we show that fragment N2 hampers malignant cells to escape the primary tumor site. This indicates that an internal portion of RasGAP, which can be released by caspase-3 cleavage, can act as a metastasis suppressor.

MATERIAL AND METHODS

Cell lines, cell culture, lentiviral infection, Western blotting and wound-healing assay

All cell lines were maintained in Dulbecco's Modified Eagle Medium as previously described.[17] Recombinant lentivirus production,[18] Western blotting and wound healing assays were performed as previously reported.[17]

Generation of stable 4T1 clones

4T1 cells were transfected with the pEGFP-C1 and pTK-Hyg (3:1 ratio) or with GFP-HA-hRasGAP[158-455] and pTK-Hyg (3:1 ratio) using the calcium phosphate method as previously reported.[17] The cells were selected using hygromycin B (selection: 200 μg/ml; maintenance: 100 μg/ml) until appearance of colonies. GFP-positive colonies were picked, and screened by microscopy and immunoblotting. Plasmids used in this study are described in the supplementary methods.

Antibody description

The antibodies used in this study were obtained from the following sources: anti-GFP (JL-8) (Clontech; ref: 632381; 1:2500), anti-RasGAP (Enzo life sciences; ref: ALX-210-860-R100; 1:250), anti-β-actin (Chemicon International Inc; ref: MAB1501; 1:5000) and anti-HA (Covence; ref: MMS-101r; 1:1000). The secondary antibodies were IRDye800-conjugated anti-mouse IgG (Rockland; ref: 610-132-121; 1:5000; milk) and AlexaFluor680-conjugated anti-rabbit IgG (Molecular Probes; ref: A21109; 1:5000; milk).

4T1 orthotopic model

We carried out orthotopic implantations as previously described[19] and under authorization license (Swiss Animal Protection Ordinance; permit number: 2379). Briefly, Balb/c female mice (Charles River) with 100'000 murine mammary cancer 4T1-derived stable clones in 20 % Matrigel (BD Biosciences; ref: 354248; diluted in PBS). The mice were sacrificed after 29 days and analyzed for the presence of lung metastases. The analyses involving TAT-RasGAP$_{317-326}$ injection are described in the supplementary methods.

Primary tumor and metastasis measurement

Tumor volumes were quantified as described earlier.[20] For metastasis analyses, five equidistant sections per lung were performed and stained with hematoxylin/eosin (H/E). The number of metastatic foci was reported as the mean of the five slides per organ and normalized to the maximal effect per experiment. The metastatic index was calculated by dividing the normalized number of metastatic foci by the corresponding mouse tumor weight (normalized to the maximal effect per experiment).

Experimental metastasis assay

Experimental metastasis assays were performed as previously reported.[21] Experimental details appear in the supplementary methods. Briefly, nude NMRI mice were injected with fragment N2 stably expressing MDA-MB-231-Luc (that stably express firefly luciferase) cells and sacrificed after 46 days. Bioluminescence of the lungs was assessed as described in the supplementary methods.

Statistical analysis

The statistical tests were achieved using the R software (version 2.11.0). The tumor growth and migration assays were analyzed by repeated measurement ANOVAs. Every metastasis and tumor size analyses were analyzed by non-parametric Mann-Whitney U tests. The Bonferroni correction was applied when more than one comparison was performed. Asterisks denote statistical differences (*: p-value < 0.05; **: p-value < 0.01 after Bonferonni corrections). Box plot description appears in the supplementary methods. Except when displayed as box plots, the results were expressed as mean +/- 95% confidence intervals.

RESULTS

To evaluate the role of fragment N2 during metastatic progression, we used the well-established 4T1 murine breast cancer model.[22] 4T1 cells, when implanted orthotopically in the mammary fat pad of syngeneic Balb/c mice, efficiently metastasize to the lungs.[22] We therefore generated several 4T1 clones expressing fragment N2 fused to a green fluorescent protein (GFP) or GFP alone as controls. Most of these clones express elevated levels of GFP and GFP-fragment N2 (from now referred to as GFP-N2) as revealed by immunoblotting (**Figure 1A**) and fluorescence microscopy (**Figure 1B**). All clones expressing fragment N2 displayed impaired motile capacity (**Figure 1C**). Of note, even the GFP-N2 #6 and #8 clones in which GFP-fragment N2 is degraded exhibited reduced migration.

Since fragment N2 efficiently inhibits migration, we evaluated its *in vivo* capacity to prevent metastasis formation to the lungs. We selected two GFP (#3 and #5) and two GFP-N2 (#9 and #10) clones to minimize the risk of generating clone-specific effects. When implanted in the mammary gland, all clones produced similarly sized primary tumors (**Figure 2A**) and of comparable weight after 29 days (**Figure 2B**). This indicates that fragment N2 does not affect tumor take and tumor growth. In contrast, the number of lung metastasis was significantly lower in mice bearing primary tumors that express fragment N2 (**Figure 2C**). Consistently, the metastatic index (number of metastasis corrected for the primary tumor weight) of the N2-expressing clones was significantly lower than the metastatic index of GFP-expressing clones (**Figure 2D**). These data indicate that expression of fragment N2 impairs metastatic progression. Noteworthily, fragment N2 did not affect the epithelial-to-mesenchymal transition (EMT), a process implicated in metastatic dissemination[23] because the levels of E-cadherin or vimentin, two hallmark molecules modulated during EMT, remained unchanged in 4T1 clones overexpressing fragment N2 (data not shown).

To determine the stability of fragment N2 expression in the 4T1 clones *in vivo*, we injected five mice with 4T1 GFP-N2 #10, sacrificed them at different time points and analyzed the expression of GFP-N2. This experiment revealed that expression of GFP-N2 was partially lost 16 days after tumor implantation and completely abrogated 22 days after implantation (**Figure 2E**). In contrast, the GFP-N2 #10 clone cultured *in vitro* did not lose fragment N2 expression during this time frame (**Figure 2E**). Loss of fragment N2 expression from tumors growing in mice may be an indication that this fragment exerts some

tumor suppressive effects. One could therefore conceive that if fragment N2 expression was not progressively disappearing during the *in vivo* metastasis development experiment, the 4T1 clones initially expressing fragment N2 would have produced much fewer metastases than those actually observed.

Cell migration is considered to contribute to metastasis at two discrete steps in the metastatic cascade: at an early metastatic stage when cells escape from the primary tumor and again at a later stage when cells extravasate and invade the parenchyma of the newly colonized site. The 4T1 *in vivo* model does not allow differentiating whether fragment N2 inhibits escape from the primary tumor, colonization of the secondary site or both. To assess whether fragment N2 inhibits the capacity of circulating tumor cells to invade tissues, the breast cancer MDA-MB-231 cell line expressing the luciferase gene and freshly infected with lentiviruses expressing or not fragment N2 were injected in the bloodstream of nude mice. Their capacity to colonize lungs was then evaluated by measuring the luciferase activity in these organs 46 days after the injection. **Figure 2F** shows that fragment N2 did not alter the capacity of MDA-MB-231 cells to invade the lungs. There was no or minimal loss of fragment N2 in the fragment N2-infected cells during the time-course of this experiment (blot of **Figure 2F**). Altogether, these experiments indicate that fragment N2 inhibits metastatic progression by preventing cancer cells from escaping the primary site but not by inhibiting colonization of distal sites.

As fragment N2 inhibits migration, a logical follow up was to assess whether the cell-permeable TAT-RasGAP$_{317\text{-}326}$ peptide was also able to inhibit metastasis formation. TAT-RasGAP$_{317\text{-}326}$, when injected intraperitoneally, was shown to accumulate in subcutaneously established HCT116 tumors and to improve the effects of chemotherapy on preventing the growth of these tumors.[20] Unfortunately, HCT116 tumors do not metastasize to other organs and hence could not be employed here to investigate the anti-metastatic potential of TAT-RasGAP$_{317\text{-}326}$. We therefore used the 4T1 model instead. However, this model is not ideal in the present setting because ^{125}I-labelled TAT-RasGAP$_{317\text{-}326}$ injected intraperitoneally failed to accumulate in primary 4T1 tumors (**supplementary Figure 1A**). Our conclusion that the peptide did not accumulate in the tumor derives from the fact that the radioactive values in the tumor were even lower than the residual signal found in the blood (**supplementary Figure 1A**). In contrast, it accumulated in the liver as shown previously (**supplementary Figure 1A**).[20] Moreover, it is impossible to test if the weakly delivered TAT-RasGAP$_{317\text{-}326}$ dose has sufficient functional effects as 4T1 cells are not sensitive to

genotoxins and could therefore not be tested for sensitization to apoptosis. We nevertheless, tested whether the RasGAP peptide could affect the ability of 4T1 cells to metastasize. Starting at the time of 4T1 tumors cells in the fat pad, mice were injected three times a week with 1.6 mg/kg TAT-RasGAP$_{317-326}$. The primary tumor size and weight were not affected by the peptide treatment (**supplementary Figure 1B-C**). Twenty-five days after tumor injection, the mice were sacrificed and the lungs were analyzed for the presence of metastases. TAT-RasGAP$_{317-326}$-treated mice did not display fewer metastatic foci (**supplementary Figure 1D**). As the peptide does not apparently accumulate in 4T1 tumors, the negative nature of these experiments do not allow us to conclude whether TAT-RasGAP$_{317-326}$ inhibits or not metastasis formation.

DISCUSSION

Cancer therapy still suffers from a lack of metastasis-specific drugs. Only a few of them are used in the clinics to treat specific cancers while the majority remains in early clinical trials.[24] Here, we provide the proof of concept that the fragment N2 of RasGAP acts as a metastasis suppressor. Hence, compounds bearing fragment N2 anti-metastatic activity may have the potential for the development of pharmacological compounds with anti-metastatic activities. We have derived a cell-permeable protease-resistant 10 amino acid peptide corresponding to a short region of fragment N2. This compound, called TAT-RasGAP$_{317-326}$, efficiently sensitizes cancer cells to various anti-tumor treatments, both *in vitro* and *in vivo*.[16,20] It also increases cell adherence, blocks cell migration and prevents invasion.[17] This TAT-RasGAP$_{317-326}$ peptide has therefore the potential to inhibit metastasis development *in vivo*. Unfortunately, the experiment we have performed here did not allow us to determine whether TAT-RasGAP$_{317-326}$ acts as a metastasis blocker because this compound failed to accumulate in breast-implanted 4T1 tumors. Thus, testing this hypothesis needs to await the development of compounds able to accumulate at sufficient levels in primary tumors and/or target organs. The prototypical issues associated with peptide therapeutics are the clearance by the liver, the weak selectivity of delivery and the peptide short half-lives. In the case of TAT-RasGAP$_{317-326}$ this last concern has been circumscribed by using D-amino acids for its synthesis but there is still room for improvement concerning the first two issues.[25,26] The development of small molecules mimicking the activity of TAT-RasGAP$_{317-326}$ is a suited alternative. We recently found that deleted in liver cancer-1 (DLC1), a RhoGAP and metastasis suppressor, was required by TAT-RasGAP$_{317-326}$ to prevent migration.[17] Work based on the interaction between fragment N2 and DLC1 could potentially lead to the development of a small molecule with TAT-RasGAP$_{317-326}$-like activities.

Fragment N2 can be produced endogenously in response to stress,[12] although probably not to the levels obtained in the clones used in the present study. One could anticipate that the endogenous fragment N2 plays some physiological roles, potentially in the context of malignant transformation. In cancer development, pre-malignant cells experience oncogenic stress that induces caspase activation leading to apoptosis in many but not all cells.[27] There are indeed cases, such as in breast cancer, where caspase-3 activity is higher in malignant tissues than in corresponding normal ones.[28] Because of caspase activation, the surviving cells may produce fragment N2 that has then the potential to exert two tumor suppressor

functions. First, fragment N2 can render cancer cells more sensitive to stress-induced death and therefore contribute to their elimination if the first wave of caspase activation failed to do so. Second, fragment N2 can prevent dissemination of surviving cancer cell hence blocking metastasis formation.

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Conflict of interest in the footnote [1]

[1] CW is a co-inventor of the TAT-RasGAP$_{317–326}$ compound as an anti-tumor agent (patent owned by the University of Lausanne) and may receive royalties from patent licensing if the compound is commercialized.

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FIGURE LEGENDS

Figure 1. Stable expression of fragment N2 inhibits cell migration.

A. Stable 4T1 clones were screened for expression of GFP and GFP-N2 by immunoblotting against GFP and RasGAP.

B. Representative images of GFP-positive 4T1 stable clones. The nucleus of untransfected 4T1 cells is also shown by Hoechst-33342 staining. Scale bar: 50 μm.

C. Stable 4T1 clones were subjected to wound-healing scratch assays. The left panel displays the progression of wound width over time for every clones (*n*=1 experiment). The right panel displays the percentage of wound closure for the clones that were selected for further experiments (*n*=4 experiments). Asterisks denote significant differences between the indicated groups after repeated measurement ANOVA.

Figure 2. Fragment N2 inhibits metastatic progression.

A-E. Balb/c mice were injected with four stable 4T1 clones expressing GFP (#3 and #5) or GFP-N2 (#9 and #10). The experiments were performed three times independently using 4-6 mice per condition in each experiment. The data were pooled per condition. The figure displays the tumor growth (**A**), the tumor weight after sacrifice (**B**), the normalized number of metastatic foci per lung (**C**), and the metastatic index (**D**). Of note, when pooling GFP clones together and GFP-N2 clones together, the GFP-N2 group exhibits a highly significant metastatic index decrease over the GFP group (p=0.00012).

E. 4T1-GFP-N2 #10 cells were implanted orthotopically (*in vivo*) or maintained in culture (*in vitro*). Immunoblotting against RasGAP was performed for the indicated time.). The ~40 kDa fragment detected at 11 days *in vitro*, and apparently in the *in vivo* condition as well, corresponds to the similarly sized fragment observed in the GFP-N2 #6 and #8 clones (see Figure 1A, upper blot). Based on the size of this fragment and the fact that it is recognized by the anti-RasGAP antibody, it can be assumed that it contains the entirety of fragment N2.

F. An experimental metastasis assay was done by injecting MDA-MB-231-Luc cells infected with fragment N2 (or its empty vector). The bioluminescence and the number of lung-invading cells are reported in the graph. Seven to eight mice were analyzed per condition. Immunoblotting against the HA tag and β-actin is displayed on the left for cells that were maintained in culture during the time-frame of the assay.

In panels B, C, D, and F, the results are shown as boxplots on the left while raw data points are displayed on the right.

SUPPLEMENTARY MATERIAL AND METHODS

Plasmid description

The extension .dn3 indicates that the backbone plasmids are pcDNA3 (#1) from Invitrogen. The **pEGFP-C1** (#6) plasmid encodes the green fluorescent protein and is from Clontech (ref: 6084-1). The **pTK-Hyg** (#317) plasmid encodes for a protein that confers resistance to the hygromycin B (Roche; ref: 12653400) and is from Clontech (ref: 6153-1). **HA-hRasGAP[158-455].dn3** (#145) previously called HA-N2.dn3 (1), encodes the HA-tagged form of fragment N2. **GFP-HA-hRasGAP[158-455]** (#213), previously named GFP-HA-N2 (2) encodes an HA-tagged version of fragment N2 fused with a GFP-protein at the N-terminal. The **HA-hRasGAP[158-455].lti** (#769) used for lentiviral infection was described earlier (3).

Experimental metastasis assay

Experimental metastasis assays were performed as previously reported (4). Nude mice were used (Janvier; ref: Rj:NMRI-$^{Foxn1nu/Foxn1nu}$) and MDA-MB-231 firefly-luciferase expressing cell were injected. Peptide regimen was similar than with 4T1 model except that it last for 30 days. Alternatively, MDA-MB-231 were infected by lentiviruses encoding the RasGAP Fragment N2 (or its empty vector), then were injected in the tail vein and sacrificed after 46 days. Lungs were taken out. The blood that was remaining in the lungs was washed by PBS perfusion into the right cardiac ventricle. The left lobes were weighed, lyzed and 20 µg of lung lysate were analyzed for luciferase activity using the GloMax luminometer and according to the manufacturer's instructions (Promega; ref: E6521). The results were reported in relative light units (R.L.U.) and in number of cells as known number of cells were quantified *in vitro* for their bioluminescence.

TAT-RasGAP$_{317-326}$ synthesis

The TAT-RasGAP$_{317-326}$ peptide (GRKKRRQRRRGGWMWVTNLRTD) was described and synthesized as previously reported (5;6).

Radioiodination of the TAT-Y-RasGAP$_{317-326}$ peptide

Ten MBq Na[I^{125}]-Iodide (Perkin Elmer) were added to 4.8 µl of 1 mM TAT-Y-RasGAP$_{317-326}$ (GRKKRRQRRRG**Y**GWMWVTNLRTD) in a glass vial coated with 100 µg iodogen (Iodination reagent; Pierce; ref: 28600). The vial was placed on ice for 20 minutes. The mixture was diluted up to 1 ml with gelatin eluting buffer (0.25 % gelatin in PBS (w/v)) and then loaded on a PD Minitrap G-10 column (GE Healthcare; ref: 28-9180-10). The radiolabeled peptide was then eluted in 0.5 ml gelatin elution buffer.

TAT-RasGAP$_{317-326}$ biodistribution

Five Balb/c mice were injected with one hundred thousand 4T1 cells in the mammary fat pads. Fourteen days after cancer cell injection, the mice were injected with 1.6 mg/kg TAT-RasGAP$_{317-326}$ peptide (containing 5 % (v/v) of I^{125}-radiolabeled peptide). Six hours after, the mice were sacrificed and the 4T1 tumor and organ radioactivity were recorded in counts per minute (cpm). Since the radioactivity of the whole mouse was recorded, the percentage of the injected dose per organ could be calculated.

4T1 orthotopic model with TAT-RasGAP$_{317-326}$

We carried out orthotopic implantations as described in the material and methods. Mice treated with the RasGAP peptide were injected intraperitoneally with 1.6 mg/kg TAT-RasGAP$_{317-326}$ (or its vehicle: PBS) three times a week (every monday, wednesday and friday) and sacrificed after 25 days.

Boxplot description

Boxplots were performed as follows: the line in a box indicates the median; the box contains 50% of values (i.e. 25% of those above and 25% of those below the median); whisker's length corresponds to 1.5 time of the box's length (if shorter, the length of the whisker reaches the lowest or the highest value of the data set). Empty circles represent outlier values (i.e. outside the range covered by the box and the whiskers).

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Figure 1

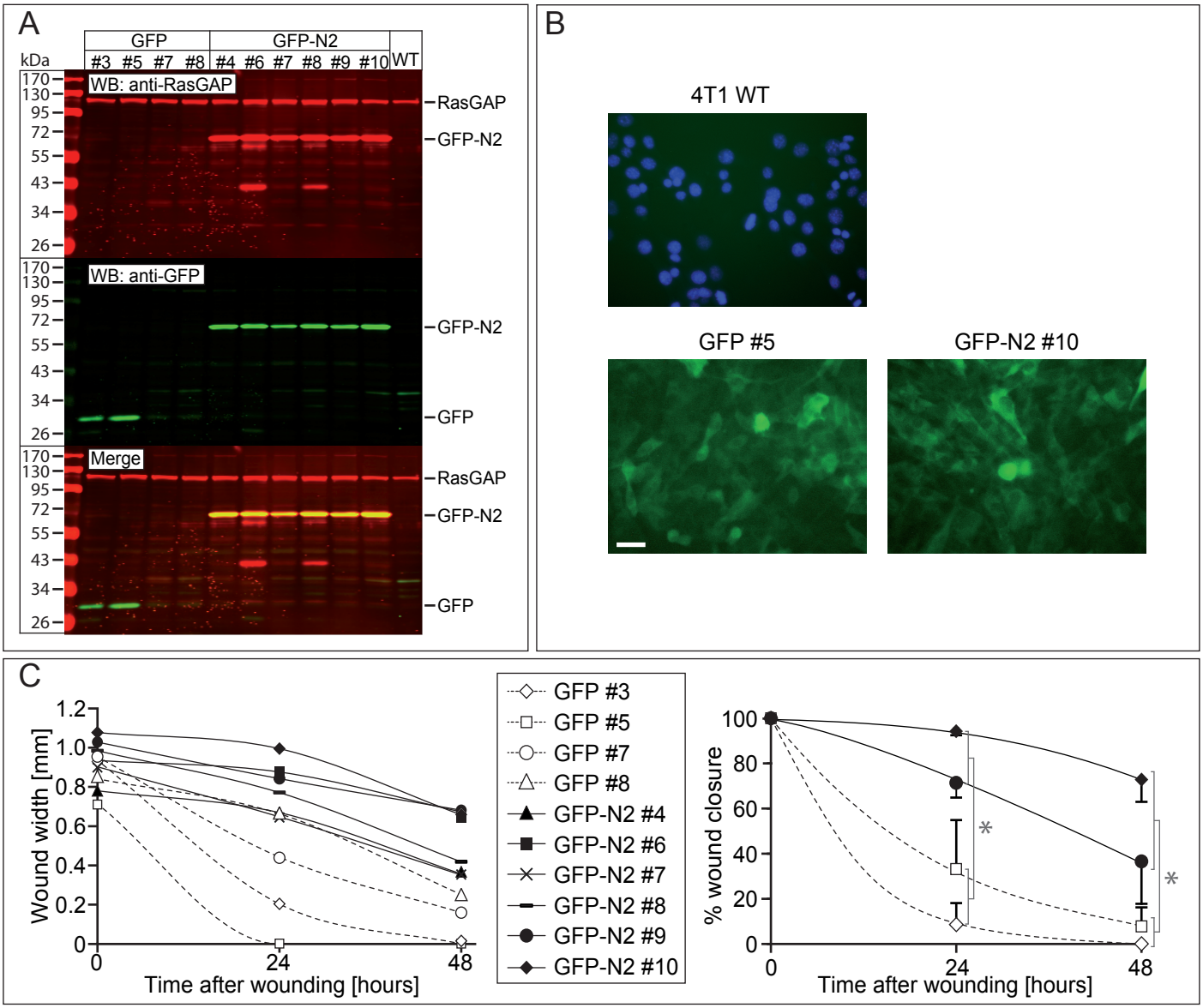
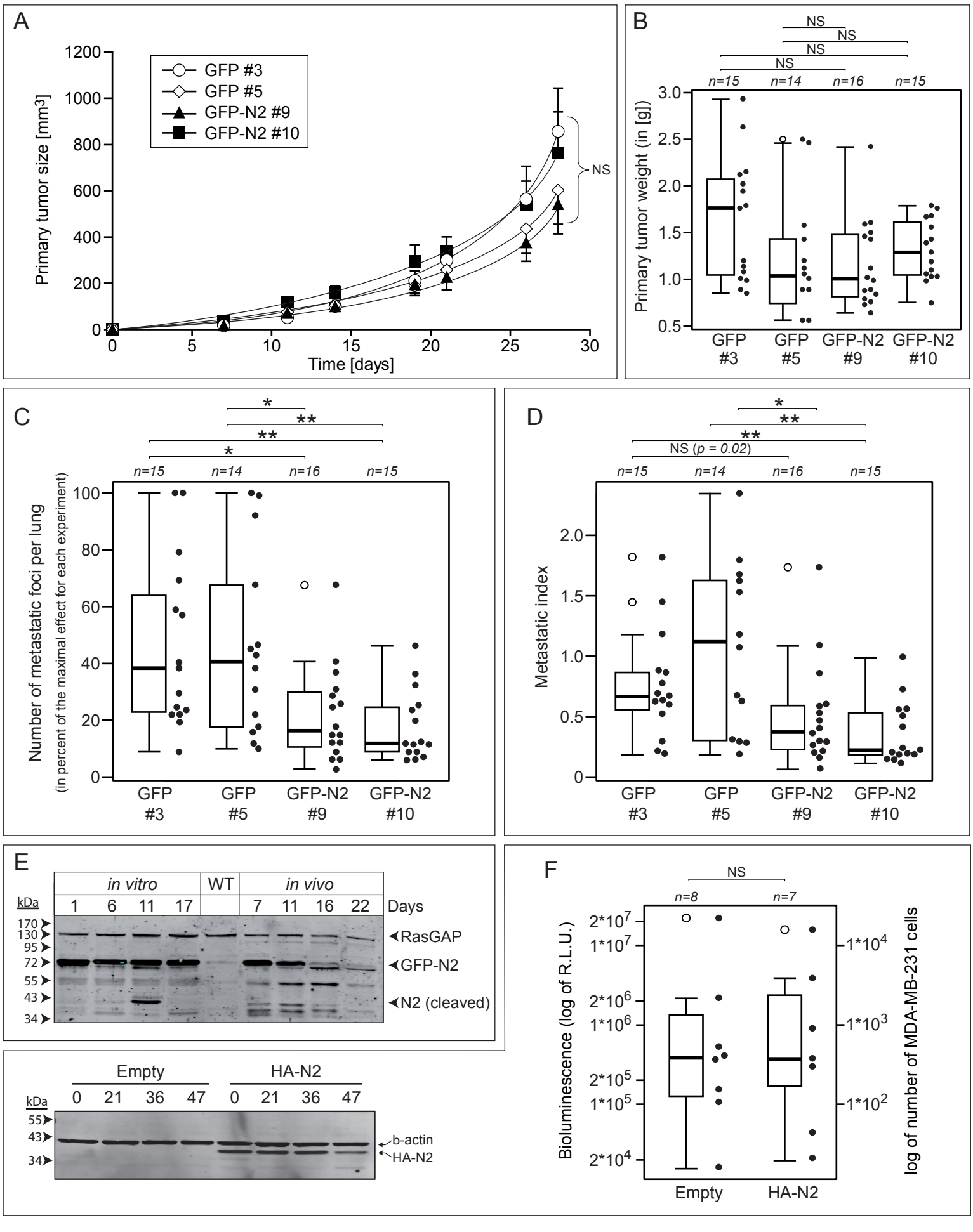
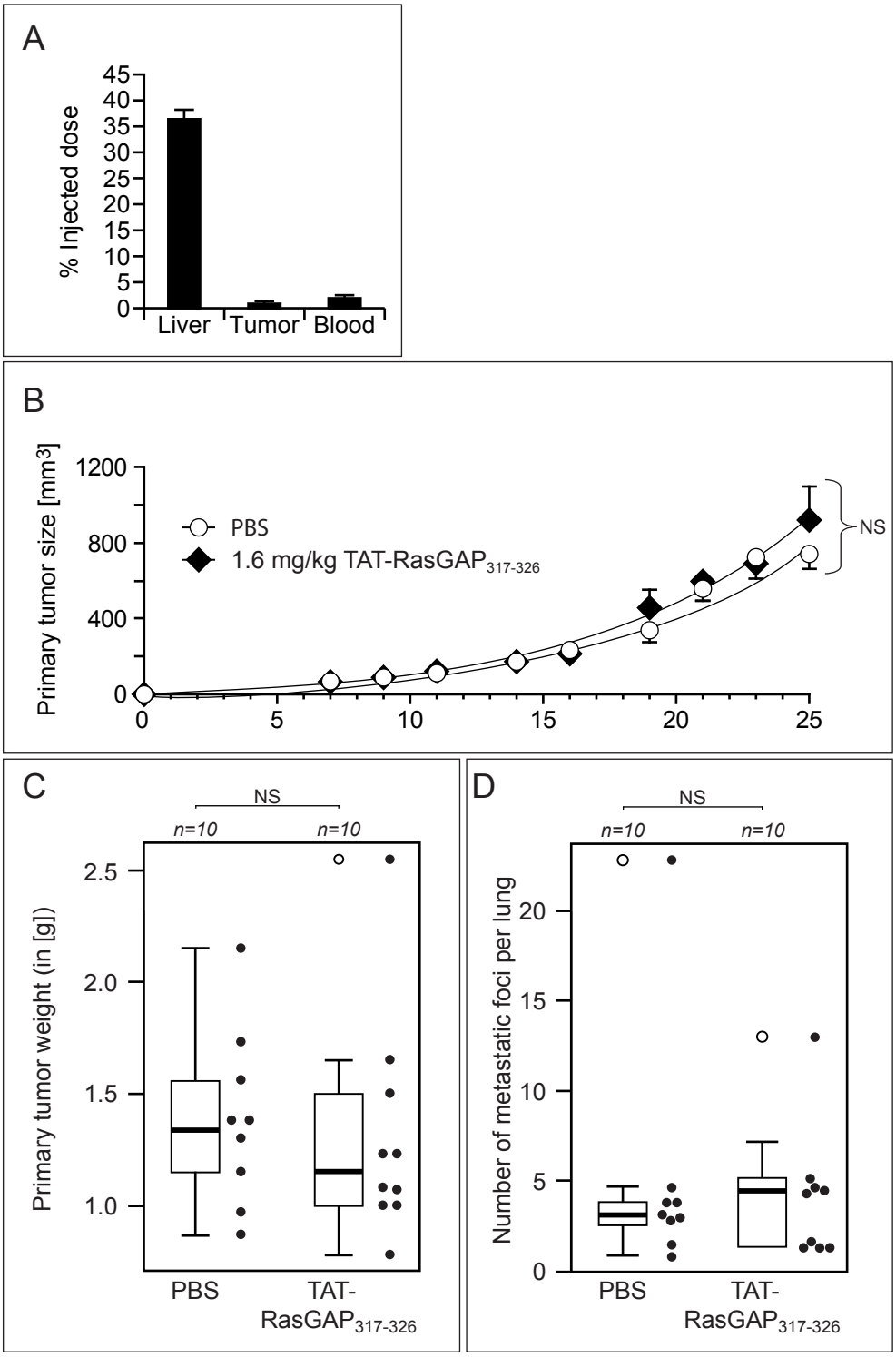

A
GFP
GFP-N2
kDa
#3 #5 #7 #8
#4 #6 #7 #8 #9 #10
WT
WB: anti-RasGAP
RasGAP
GFP-N2
WB: anti-GFP
GFP-N2
GFP
Merge
RasGAP
GFP-N2
GFP
170
130
95
72
55
43
34
26
B
4T1 WT
GFP #5
GFP-N2 #10
C
Wound width [mm]
Time after wounding [hours]
GFP #3
GFP #5
GFP #7
GFP #8
GFP-N2 #4
GFP-N2 #6
GFP-N2 #7
GFP-N2 #8
GFP-N2 #9
GFP-N2 #10
% wound closure
Time after wounding [hours]
*
*

Figure 2


A
Primary tumor size [mm3]
Time [days]
GFP #3
GFP #5
GFP-N2 #9
GFP-N2 #10
NS
B
Primary tumor weight (in [g])
n=15
n=14
n=16
n=15
NS
GFP #3
GFP #5
GFP-N2 #9
GFP-N2 #10
C
Number of metastatic foci per lung
(in percent of the maximal effect for each experiment)
D
Metastatic index
NS (p = 0.02)
E
in vitro
WT
in vivo
kDa
Days
RasGAP
GFP-N2
N2 (cleaved)
Empty
HA-N2
b-actin
F
Bioluminescence (log of R.L.U.)
log of number of MDA-MB-231 cells
n=8
n=7

Supplementary Figure 1



Supplementary Figure 1. Effect of TAT-RasGAP$_{317-326}$ on 4T1 metastasis formation.
(**A**) The biodelivery of 1.6 mg/kg I^{125}-labelled TAT-RasGAP$_{317-326}$ in Balb/c bearing pre-established 4T1 tumors was reported in percentage of the injected dose (n = 5 mice).
(**B-D**) Balb/c mice were injected with 4T1 cells and then treated three times a week with 1.6 mg/kg TAT-RasGAP$_{317-326}$ for 25 days. The figure displays the tumor growth (**B**), the tumor weight after sacrifice (**C**) and the number of metastatic foci per lung (**D**). Ten mice per condition were analyzed. The results are shown as boxplots on the left while raw data points are displayed on the right.