Alternatively spliced fibronectin extra domain A is required for hemangiogenic recovery upon bone marrow chemotherapy

Alessandro Malara,[1,2] Cristian Gruppi,[1] Giuseppe Celesti,[3] Vittorio Abbonante,[1,2] Gianluca Viarengo,[4] Luigi Laghi,[3] Luigi De Marco,[5,6] Andrés F. Muro[7] and Alessandra Balduini,[1,2,8]

[1]Department of Molecular Medicine, University of Pavia, Italy; [2]Laboratory of Biotechnology, IRCCS San Matteo Foundation, Pavia, Italy; [3]Laboratory of Molecular Gastroenterology, Humanitas Clinical and Research Center, Rozzano, Milan, Italy; [4]Immunohaematology and Transfusion Service, Apheresis and Cell Therapy Unit, IRCCS San Matteo Foundation, Pavia, Italy; [5]Department of Translational Research, National Cancer Center (IRCCS CRO), Aviano, Italy; [6]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA; [7]The International Centre for Genetic Engineering and Biotechnology, Trieste, Italy and [8]Department of Biomedical Engineering, Tufts University, Medford, MA, USA.

Correspondence: alessandra.balduini@unipv.it
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Alessandro Malara[1,2], Cristian Gruppi[1], Giuseppe Celesti[3], Vittorio Abbonante[1,2], Gianluca Viarengo[4], Luigi Laghi[3], Luigi De Marco[5, 6], Andrés F. Muro[7] and Alessandra Balduini[1, 8].

Affiliations: [1] Department of Molecular Medicine, University of Pavia, Pavia, Italy. [2] Laboratory of Biotechnology, IRCCS San Matteo Foundation, Pavia, Italy. [3] Laboratory of Molecular Gastroenterology, Humanitas Clinical and Research Center, Rozzano, Milan, Italy. [4] Immunohaematology and Transfusion Service, Apheresis and Cell Therapy Unit, IRCCS San Matteo Foundation, Pavia, Italy. [5] Department of Translational Research, National Cancer Center (IRCCS CRO), Aviano, Italy. [6] Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA. [7] The International Centre for Genetic Engineering and Biotechnology, Trieste, Italy. [8] Department of Biomedical Engineering, Tufts University, Medford, MA, USA.

Materials and Methods

Animals

C57BL/6 wild type mice were from Charles River Laboratories, Italy. EIIIA+/+ and EIIIA-/- mouse strains were previously generated (Muro*, et al* 2003). Mice were housed at the animal facility of the Department of Physiology, section of General Physiology, University of Pavia (approval # 3/2013, 19/11/2013). All animals were sacrificed according to the current European legal Animal Practice requirements.

In vivo treatments

Mice were injected with 150mg/Kg body weight of 5 fluorouracil (5-FU) intra peritoneally (i.p.) as previously described (Malara*, et al* 2014). Age paired mice were always injected with PBS as control. Differential blood cell counts were performed on a Cell Dyn 3700 hematology analyzer (Abbott Diagnostic, Illinois, USA). In vivo BrdU incorporation was performed according the protocol of An et al. (An, *et al* 2013). Vascular leakage was measured in BM fluids after injection of 100µl of 100kDa fluorescein isothiocyanate-conjugated dextran (FITC-dextran, 25 mg/ml in PBS) in the tail vein of mice two hours prior their sacrifice during 5-FU treatment. FITC-dextran was quantified in cell-free BM fluids with a fluorescence spectrophotometer at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Standard curves to calculate FITC-dextran concentration in the BM fluid samples were prepared from dilutions of FITC-dextran in PBS.

Antibodies and reagents

5-Fluorouracil was from Sigma Aldrich (Milan, Italy). The following antibodies were used: anti rat FN was from Chemicon (Merck-Millipore, Milan, Italy), anti cellular FN 3E2, anti β-actin, anti α-SMA were all from Sigma Aldrich. Anti TLR4 (clone 25 and M-300) were from Santa Cruz Biotechnology Inc. Anti VEGFR-3 antibody was from eBioscience. Anti phospho-NF-κβ (Ser536) and total NF-κβ (E498) were purchased from Cell Signaling Technologies. FITC-Brdu Flow Kit was purchased from Beckton Dickinson (Milan, Italy).

Western blotting

BM cells were lysed in hepes-glycerol lysis buffer (Hepes 50mM, NaCl 150 mM, 10% glycerol, 1% triton x-100, MgCl2 1,5mM, EGTA 1mM) containing leupeptin 1µg/ml, aprotinin 1µg/ml, for 30 minutes at 4 °C. Samples were clarified by centrifugation at 15700g at 4 °C for 15 minutes. Laemmli sample buffer was then added to supernatants. BM pellets were solubilized using 500 µl DOC lysis buffer (PBS, deoxycholic acid 0.1%, EDTA 2mM). After centrifugation, the DOC-insoluble pellet was solubilized in 50 µl of 2% SDS, 20 mM Tris-HCl, pH 8.8, 2 mM PMSF, 2 mM iodoacetic acid, 2 mM N-ethylmaleimide and 2 mM EDTA. Samples were always heated at 95°C for 3 minutes, separated by electrophoresis on 8% or 12% sodium dodecyl sulfate-polyacrylamide gel and then transferred to polyvinylidine fluoride membranes. Membrane were probed

with primary antibodies, washed 3 times with PBS and Tween 0.1% and incubated with an appropriate peroxidase-coniugate secondary antibody. Membrane were visualized using Immobilon western chemiluminescent HRP substrate (Millipore, Milan, Italy) and ChemiDoc XRS Imaging System (BioRad Laboratories Inc, Milan, Italy).

Enzyme-linked Immunosorbent Assays (ELISA)

BM cells were obtained by flushing mice femurs with 1ml of PBS. Cells were centrifuged at 15700 g and cell free BM fluids collected and protein concentrations were quantified in each sample by BCA assay. Samples were then used for determination of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) with commercially available mouse ELISA kits according to the manufacturer's instructions (Biolegend, London, UK). Data obtained by ELISA were normalized to total protein concentrations determined using the BCA assay.

RT-PCR and qRT-PCR

Retrotranscription (RT) was performed in a final volume of 20 µl reaction using the iScriptTM cDNA Synthesis Kit according to manufacturer instructions (BioRad Laboratories Inc, Milan, Italy). For quantitative Real Time PCR, RT samples were diluted up to 60 µl with ddH2O and 3 µl of the resulting cDNA was amplified in triplicate in 15 µl reaction mixture with 200 nM of each specific primer and SsoFast™ Evagreen® Supermix (Bio-rad Laboratories, Milan, Italy) at 1x as final concentration. The amplification reaction was performed in a CFX Real-time system (BioRad Laboratories Inc., Milan, Italy) as follows: 95°C for 5 minutes, followed by 35 cycles at 95°C for 10 seconds, 60°C for 15 seconds, 72°C for 20 seconds. Primers compassing the specific EIIIA exon of FN were as follows: forward 5'-CCCTAAAGGACTGGCATTCA-3' and reverse 5'-CATCCTCAGGGCTCGAGTAG-3'. Pre-designated KiCqStart™ primers for β2microglobulin, NOX4 and $p22^{phox}$ (CYBA) gene were purchased from Sigma-Aldrich (Milan, Italy). The BioRad CFX Manager® software 3.0 was used for the normalization of the samples (BioRad Laboratories Inc. Milan, Italy). β2-microglobulin gene expression was used for the comparative concentration analysis.

Flow cytometry

For cell immunophenotyping, the following antibodies were used: anti mouse CD3ε-FITC (clone 145-2C11), anti mouse CD8a-PE (clone 53-6.7), CD4-FITC (clone GK1.5), CD11b-PE (Mac-1, clone M1/70), Ly6G-Ly6C-FITC (Gr-1, clone RB6-8C5), anti mouse/human CD45R-PE (B220, clone RA3-6B2) (all from Biolegend, London, UK). For the analysis of BM progenitors, BM red blood cells lysed with ammonium chloride and stained with FITC-lineage cells (Miltenyi Biotech), PE-CY7 anti mouse Ly-6A/E (Sca-1, clone D7), APC-eFluor 780 anti-mouse CD117 (c-Kit, clone 2B8) (Biolegend, London, UK), PE anti mouse CD34 (clone RAM34) (BD Pharmingen, Milan, Italy) for 30 minutes and analyzed.

In vivo BrdU incorporation in hematopoietic subsets was performed according manufacturer instructions. Lineage negative cells were selected with the Lineage Cell Depletion Kit (Miltenyi Biotech) and BrdU incorporation was analyzed in LSKs (lineage negative/Sca-1+/CD117+ cells), LT-HSCs (LSK $CD34^{low/neg}$) and ST-HSCs plus MMPs population (LSK $CD34^{pos}$). FITC Anti Mouse CD45 (clone 30F11), APC anti mouse CD31 (clone 390) and APC anti CD140a (PDGFR-α, clone APA5) were all from Miltenyi Biotech (Milan, Italy). FITC anti mouse Ter119 (clone Ter119) was from eBioscience. Purification of bone marrow cell fractions was performed with microbeads-conjugated anti mouse CD45, Ter119, CD31 and CD140a according to manufacturer instructions (Miltenyi Biotech). For measurements of ROS production and NO levels by flow cytometry, BM cells were incubated 30 minutes at 37°C with 10µM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) and 5 µM DAF-FM Diacetate (4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate) (Thermo Fischer Scientific), respectively. Cells were then stained with PE-CD45, PE-Ter119 (Biolegend) and APC CD31 (Miltenyi Biotech) antibodies and analyzed.

Mks were quantified using a rat PE/CY7 anti mouse CD45 (clone 30-F11) and a rat PE anti mouse CD41 (MwReg30). Mk output was calculated as the percentage of SSC/FSC high, CD45+/CD41+ cells and normalized to the total number of CD45+ cells. All the samples were acquired with a Beckman Coulter FacsDiva flow cytometer (Beckman Coulter Inc, Milan, Italy). Non-stained samples and relative isotype controls were used to set the correct analytical gating. Off-line data analysis was performed using Beckman Coulter Kaluza® version software package.

Zymography

Briefly, supernatants from saline or 5-FU treated flushed BM cells were analyzed for protein content by bicinchoninic acid assay and equal amount of soluble protein were separated by SDS (10%)-PAGE copolymerized with 0.1% of denatured type I collagen from bovine tendon, purified as previously described (Malara et al., 2011). Gels were washed in 2.5% Triton X-100 and then incubated overnight in developing buffer. After Coomassie blue staining, gels were destained, and digital images were acquired by scanning the zymography gels with a flatbed scanner. Active human full length MMP-9 and human full length MMP-2 (10–20 ng) (Abcam, Cambridge, UK) were used as control.

Tissue collection

Femurs were removed from 6-8 weeks old mice, fixed for 24 hours in paraformaldheyde (PFA) 3% or buffered formalin 4%. Bones were decalcified in a solution of EDTA 10%, in Phosphate-Buffered Saline (w/o Calcium and Magnesium) (PBS) pH 7.2, for 2 weeks at 4°C and then paraffin embedded. 5µm-tissue sections were taken by using a Microm Microtome HM 250 (Bio Optica S.p.A, Milan, Italy) and processed for immunohistochemistry.

Immunohistochemistry

For immunohistochemistry staining, after paraffin removal and rehydration, 5µm–thick sections were treated with EDTA PH 9 antigen retrieval solution in an antigen retrieval bath or in a Decloaking chamber with a Diva Decloaker antigen retrieval solution (BioCare Medical, Concord, CA), incubated with 3% H2O2 for 15minutes, and then incubated for 1 hour at room temperature with primary antibodies. Primary antibodies dilutions were as follow: anti TLR4 1:200, anti FN 1:500, anti VEGFR-3 1:50. Specimens were then incubated with Mouse-on-Mouse HRP-Polymer Kit or MACH 4 Universal HRP-Polymer or Rat-on-Mouse HRP-Polymer Kit (BioCare Medical) according to the species of origin of the primary antibody. 3, 3'-diaminobenzidine tetra-hydrochloride (BioCare Medical) was used as chromogen, and nuclei were counterstained with Hematoxylin.

Immunoprecipitation

Co-immunoprecipitation of endogenously expressed proteins was performed on BM mononuclear cells recovered from femurs of saline or 5-FU treated mice. Cells were harvested in hepes-glycerol lysis buffer and extracts were incubated overnight with 2.5 µg of anti-TLR4 (M-300, Santa Cruz Biotechnology, Heidelberg, Germany) antibody in the presence of Protein A/G PLUS agarose beads (Santa Cruz Biotechnology, Heidelberg, Germany) and resulting complexes were washed, denatured and eluted according to the manufacturer's instruction. Mouse anti TLR4 (25, Santa Cruz Biotechnology, Heidelberg, Germany) was used to highlight TLR4 immunoprecipitation and anti EIIIA antibody FN3E2 (Sigma Aldrich, Milan, Italy) was used to detect co-immunoprecipitated FN.

Statistics

Values are expressed as mean ± SD. One way ANOVA and two way ANOVA followed by a Bonferroni post-test were used to analyze experiments. Kaplan Meier survival curve was analyzed by a Mantel Cox test. P value statistically significant were expressed as *$p<0.05$, **$p<0.01$ and ***$p<0.001$ respectively.

Supplementary References:

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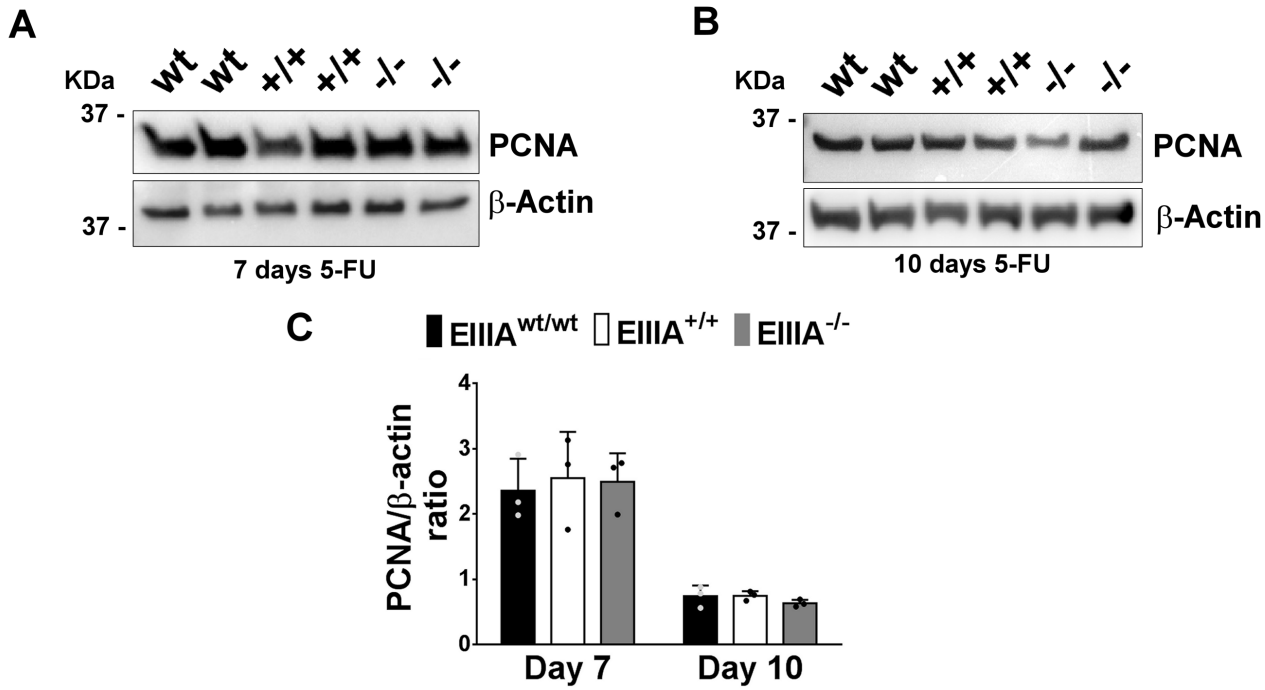
Muro, A.F., Chauhan, A.K., Gajovic, S., Iaconcig, A., Porro, F., Stanta, G. & Baralle, F.E. (2003) Regulated splicing of the fibronectin EDA exon is essential for proper skin wound healing and normal lifespan. *J Cell Biol,* **162,** 149-160.

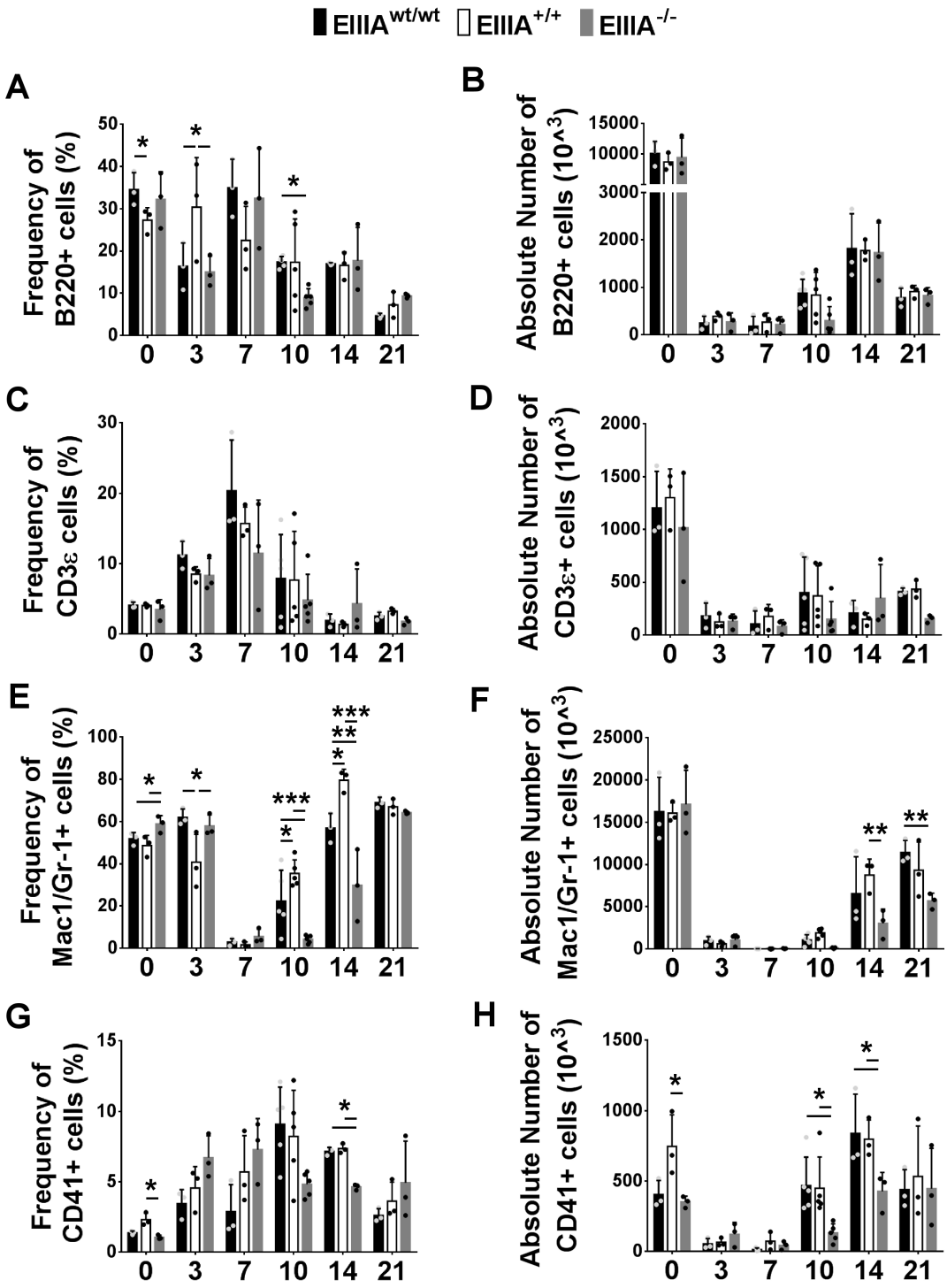
SUPPLEMENTARY FIGURES AND LEGENDS:

SUPPLEMENTARY FIGURE 1



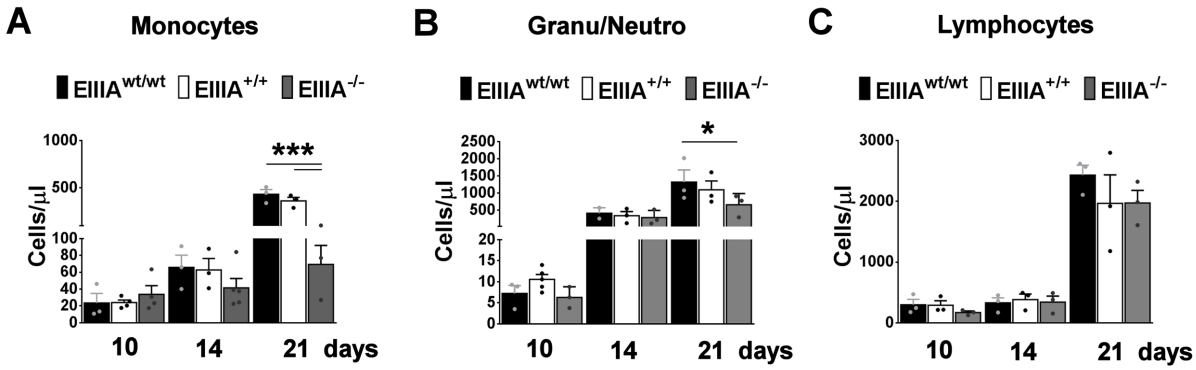
Supplementary Figure 1: A-B) Evaluation of PCNA expression in BM cells of wild type mice, EIIIA+/+ and EIIIA-/- at day 7 **(A)** and 10 **(B)** after 5-FU administration. **C)** Relative protein quantification on β-actin expression.

SUPPLEMENTARY FIGURE 2



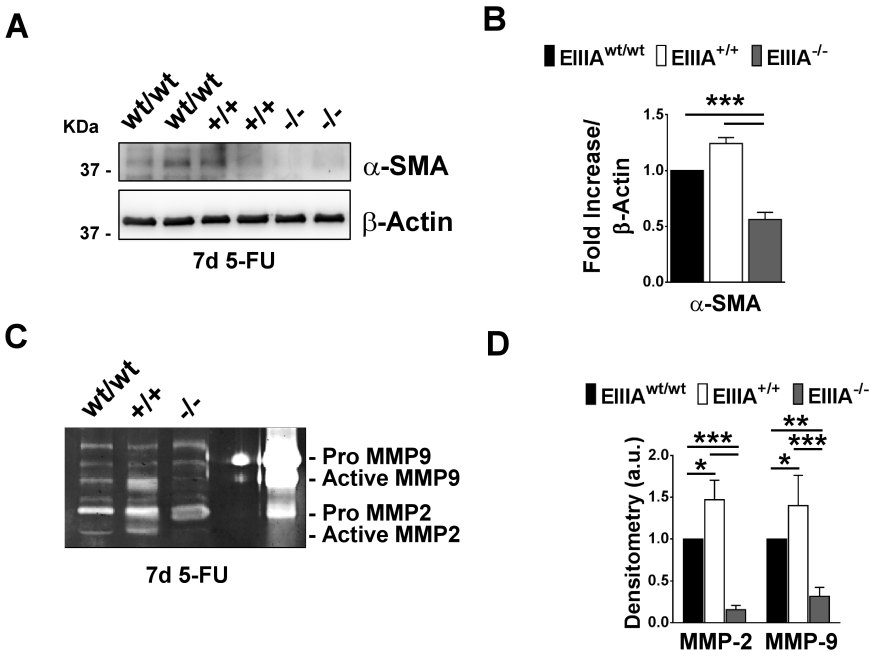
Supplementary Figure 2: Immunophenotyping of BM cells by FACS in WT mice, EIIIA+/+ and EIIIA-/- at day 0, 3, 7, 10, 14 and 21 after 5-FU administration. **A-B)** Frequencies **(A)** and absolute numbers **(B)** of B220+ cells in WT mice, EIIIA+/+ and EIIIA-/- at day 0, 3, 7, 10, 14 and 21 after 5-FU administration. **C-D)** Frequencies **(C)** and absolute numbers **(D)** of CD3ε+ cells. **E-F)** Frequencies **(E)** and absolute numbers **(F)** of CD11b+/Gr1+ cells. **G-H)** Frequencies **(G)** and absolute numbers **(H)** of CD41+ cells. At least three mice per genotype were analyzed in each experimental time point. *p<0.05, **p<0.01 and ***p<0.001, respectively.

SUPPLEMENTARY FIGURE 3



Supplementary Figure 3: Peripheral leukocyte count recovery during 5-FU treatment. A-C) Differential blood cell counts of monocytes **(A)**, Granu/Neutrophils **(B)** and Lymphocytes **(C)** in WT, EIIIA+/+ and EIIIA-/- mice at day 3, 7, 10, 14 and 21 after 5-FU administration. At least three mice per genotype were analyzed in each experimental time point. *p<0.05 and ***p<0.001, respectively.

SUPPLEMENTARY FIGURE 4



Supplementary Figure 4: Reduced stromal activity during BM recovery in mice lacking EIIIA domain of fibronectin. A-B) Western blot analysis of α-SMA expression in BM cells of WT, EIIIA$^{+/+}$ and EIIIA$^{-/-}$ mice at day 7 of 5-FU treatment **(A)** and relative densitometric analysis **(B)** normalized with β-actin. At least three independent experiments were performed. **C-D)** In situ gelatin zymography of BM supernatants in WT, EIIIA+/+ and EIIIA-/- mice after 7 days of 5-FU treatment **(C)** and relative densitometric analysis **(D)**. Purified active MMP9 and pro-MMP-2 were loaded as control in the last lane. All data are expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.