**Supplementary Figure legends:**

**Supplementary Figure 1.** A and B) The maximum tolerated dose (MTD) of cisplatin does not induce liver or organ toxicity. Serum levels of ALT and AST (U/L) (A) and blood cell counts (B) in TC-1 tumor-bearing mice 4 days after administration of 4 or 10 mg/kg cisplatin. Data are displayed as mean + SEM, 5 mice per group from one experiment. Significance was determined by Mann-Whitney test (ns = not significant).CD8 depleting antibody effectively depletes CD8+ T cells but not CD8+ dendritic cells.C) Representative flow cytometry plots of CD3+CD8+ T cells in TC-1 tumor-bearing mice 1 day after treatment with anti-CD8 mAb (clone 2.43, right panel) compared to an untreated (left panel) mouse. Numbers indicate the percentage of CD3+CD8+ T cells. D) Representative flow cytometry plots of CD8+ splenic dendritic cells in TC-1 tumor-bearing mouse 4 days after treatment with anti-CD8 mAb (right panel) compared to an untreated mouse (left panel). Numbers indicate the percentage of CD8+CD24+ cells gated on live singlet CD3-B220-CD11c+ cells.

**Supplemental Figure 2.** Induction of tumor-specific T-cell responses are dependent on cisplatin treated tumor cells. C57BL/6 mice were injected s.c with 1 × 105 TC-1 tumor cells in the flank and treated on day 8 with 10 mg/kg cisplatin or left untreated. A) Flow cytometry plots of IFN-γ producing splenic CD8+ T cells of naive (non tumor-bearing) and cisplatin-treated TC-1 or MCA205 tumor-bearing mice (day 60 post tumor challenge). IFN-γ production in splenic CD8+ T cells was determined after 4 days *in vitro* stimulation with irradiated tumor cells or left unstimulated as described in Supplementary Materials and Methods. B) On day 21 post tumor challenge, naive, untreated and cisplatin-treated mice (cisplatin-treated day 21) were sacrificed. At the same day, cisplatin-treated mice that survived tumor challenge and remained tumor free until day 60 post tumor challenge (cisplatin-treated day 60) were also sacrificed. IFN-γ and TNF production in splenic CD8+ T cells was determined after 4 days *in vitro* stimulation with irradiated TC-1 tumor cells or left unstimulated. The percentage of IFN-γ+ cells within the CD3+CD8+ T cells are normalized to non-stimulated conditions. C) Flow cytometry plots of IFN-γ producing CD8+ splenic T cells of cisplatin-treated non-tumor-bearing mice (upper two plots) and cisplatin-treated C3 tumor-bearing mice (lower four plots). Wild-type C57BL/6 mice were injected s.c with 5 × 105 C3 tumor cells in the flank or kept unchallenged. Mice were treated on day 14 with 10 mg/kg cisplatin. On day 60 post tumor challenge, all mice were sacrificed. On that day, 50% of the cisplatin-treated C3 tumor-bearing mice were cured. Spleens were dissected and single cell suspensions were stimulated overnight with irradiated C3 tumor cells in presence of brefeldin A. Next, cells were stained intracellular for IFN-γ. Numbers indicate the percentage of IFN-γ+ cells within CD3+CD8+ cells.

**Supplementary Figure 3.** Cisplatin-induced cell death enhances T-cell activation by upregulating CD70, CD80 and CD86 costimulatory molecules. A) Representative flow cytometry plots for CD8+ T-cell proliferation. Shown is the CFSE level in isolated CD8+ pmel-1 cells incubated with medium control or TC-1 tumor cells without any treatment or treated with 1.0 µg/ml cisplatin in the presence of D1 dendritic cells and long peptide as described in Supplementary Materials and Methods. The numbers show the percentage of proliferated cells. Data shown is representative of three independent experiments. B) Shown is the correlation of IFN-γ production (ng/ml) and CFSE level of pmel-1 CD8+ cells in the presence of D1 cells and TC-1 tumor cells treated with different dosages of cisplatin with the geometric mean of CFSE at each concentration. Data shown is representative of three independent experiments. C) Percentage of survival of TC-1 tumor cells upon exposure to cisplatin (µg/ml) as measured by an MTT assay. Percentages of viable cells were calculated based on the OD of each condition compared to untreated sample and expressed as percentage. Dotted lines show the concentrations that inhibited the proliferation of cells for 50% and 20%. Data shown is representative of three independent experiments. D) Expression of the costimulatory molecules 4-1BBL, OX40L and ICOSL on D1 cells in the presence of cisplatin-treated tumor cells. TC-1 tumor cells (7,000 cells/well) were incubated overnight with the indicated doses of cisplatin. Tumor cells were washed and D1 cells were added. Expression of the costimulatory molecules was measured after 72 hours incubation. Data shown is representative of three independent experiments. E) Representative flow cytometry plots for CD70, CD80 and CD86 on TC-1 and C3 tumor cells and D1 cells upon direct exposure to cisplatin. In brief, tumor cells (7,000 cells/well) and D1 cells (50,000 cells/well) were cultured in a 96-well culture flat-bottom plate. After 3 hours, cells were treated with 1 µg/ml cisplatin and incubated overnight. Then, cells were thoroughly washed and expression of CD70, CD80 and CD86 was measured on these cells by flow cytometry. F and G) Expression of CD70, CD80 and CD86 on D1 cells in the presence of IFNα with and without IFNAR blocking antibody. D1 cells were incubated for 24 hours with 1.3 µg/ml of IFNα (F) or for 72 hrs with 1.3 µg/ml IFNα and 20 µg/ml anti-IFNAR blocking antibody (G), after which the costimulatory molecule expression was determined by flow cytometry. LPS + anti-CD40 was used as a positive control and medium alone as a negative control. H) Expression of CD70, CD80 and CD86 on D1 cells with and without anti-IFNAR blocking antibody. D1 cells were incubated for 72 hours in the presence of cisplatin-treated (2.5 µg/ml) or untreated TC-1 tumor cells with and without 20 µg/ml anti-IFNAR blocking antibody, after which the costimulatory molecule expression was determined by flow cytometry.

**Supplemental Figure 4.** Categorizations of proteins released upon cisplatin treatment of tumor cells. A) Venn diagram of protein profile overlap between cisplatin-treated and untreated tumor cells. Supernatants of untreated TC-1 tumor cells or treated with 1 or 2.5 µg/ml cisplatin were measured by mass spectrometry. Numbers indicate the number of proteins in each area. B) Categorizations of cisplatin-treated TC-1 tumor cells protein profile. Supernatant of untreated and treated TC-1 tumor cells with 1 µg/ml cisplatin were measured by mass spectrometry as described in Supplemental materials and methods. Proteins with ratio of 3 or higher compared to untreated control were categorized based on their biological process according to PANTHER (Protein Analysis Through Evolutionary Relationships) database classification system.

**Supplemental Figure 5.** Modulation of costimulatory molecule expression by HMGB1.A) Expression of CD70, CD80 and CD86 on D1 cells in the presence of HMGB1. D1 cells were incubated for 4 days with 20 ng/ml of HMGB1, after which the expression of CD70, CD80 and CD86 was determined by flow cytometry. LPS + anti-CD40 was used as a positive control and medium alone as a negative control. B) Expression of CD70, CD80 and CD86 on wild-type or *Tlr4-/-*BMDCs in the presence of TC-1 tumor cells treated with 2.5 µg/ml cisplatin. The cells were incubated for 72 hours, after which the expression of CD70, CD80 and CD86 was determined by flow cytometry. C) Survival of TC-1 tumor-bearing wild-type mice following treatment with cisplatin and HMGB1 neutralizing antibody (anti-HMGB1). A schematic diagram of the therapy regimen is displayed at the top. Experiment is performed once with 4 mice in the untreated and anti-HMGB1 groups and 7-9 mice in the cisplatin treated groups with and without treatment with anti-HMGB1. D) Survival of TC-1 tumor-bearing wild-type and *Tlr4-/-* mice following treatment with cisplatin. A schematic diagram of the therapy regimen is displayed at the top. Experiment is performed once with 4-5 mice in the untreated groups and 6-7 mice in the cisplatin treated.

**Supplemental Figure 6.** Effects of cisplatin on the tumor microenvironment.Wild-type C57BL/6 mice were injected s.c with 1 × 105 TC-1 or 5 × 105 C3 tumor cells in the flank (day 0). On day 14 (TC-1 model) or day 20 (C3 model), mice were treated systemically with 10 mg/kg cisplatin. Four days later, tumors were dissected and analysed by flow cytometry. A) Tumor sizes of cisplatin treated and untreated mice on day 4 after cisplatin in TC-1 and C3 tumor models. Data is expressed as the mean with SEM. Significance was determined by a Mann Whitney test. B) Representative flow cytometry plots of leukocytes (CD45+) within the live gate on day 18 for the TC-1 (upper panels) and day 24 for C3 (lower panels) tumors of untreated and cisplatin-treated animals. C) Representative flow cytometry plot of intratumoral CD25 and FoxP3 cells gated on CD45+CD4+classII- cells. Percentage of intratumoral CD25+ of CD4+ cells in TC-1 and C3 tumor model analysed 4 days after cisplatin treatment. D) The expression of CD11c on the four myeloid subsets isolated from TC-1 tumor. Each dot represents data from an individual mouse. Data is expressed as the mean with SEM. Significance was determined by a Mann Whitney test. \*, *P*<0.05, \*\*, *P*<0.01, \*\*\*, *P*<0.001. E) The percentage of CD11cint and CD11chi of Ly6ChiF4/80hiCD11b+ myeloid cells isolated from TC-1 tumor. Each dot represents data from an individual mouse. Data is expressed as the mean with SEM. Significance was determined by a Mann Whitney test. \*, *P*<0.05, \*\*, *P*<0.01, \*\*\*, *P*<0.001. F) Wild-type C57BL/6 mice were injected s.c with 1 × 105 TC-1 tumor cells in the flank and treated on day 8 with 4 or 10 mg/kg cisplatin or left untreated. After 4 days, MCP-1 levels were measured in serum of mice by cytokine multiplex assays. G) Representative flow cytometry plot of MDSCs, based on gating on CD11b and Gr-1 expression, in untreated and cisplatin-treated mice. Graphs indicate the percentage of intratumoral CD11b+Gr-1hi, CD11b+Gr-1int and CD11b+Gr-1hi analysed 4 days after cisplatin treatment in TC-1 tumor model.

**Supplemental Figure 7.** Cisplatin treatment does not affect the expression of costimulatory molecules on tumor cells, total myeloid cells of spleen and TDLN *in vivo*.C57BL/6 mice were injected s.c with 1 × 105 TC-1 or 5 × 105 C3 tumor cells in the flank. On day 14 (TC-1 model) or day 20 (C3 model), mice were treated systemically with 10 mg/kg cisplatin. Four days later, tumors were dissected and analysed by flow cytometry. A) The expression of CD70, CD80 and CD86 on CD11cint and CD11chi cells of Ly6ChiF4/80hi CD11b+ myeloid cells isolated from C3 tumor. Each dot represents data from an individual mouse. Data is expressed as the mean with SEM. Significance was determined by a Mann Whitney test. \*, *P*<0.05; \*\*, *P*<0.01; \*\*\*, *P*<0.001. B and C) Expression of CD70, CD80 and CD86 on total myeloid cells in spleens (B) and tumor draining lymph nodes (TDLN) (C) of TC-1 tumor-bearing mice. The expression of CD70, CD80 and CD86 was determined on total myeloid cells gated on 7AAD-CD45+CD3-CD11b+ cells in spleens and TDLN of TC-1 tumor-bearing mice. Upper panels show the representative flow cytometry plots (samples closest to median value) for CD70, CD80 and CD86 on total myeloid cells in spleens and TDLNs of untreated mice (solid black lines), cisplatin-treated mice (grey histograms). Lower panels show the expression of CD70, CD80 and CD86 on the total myeloid cells in spleens (B) and TDLN (C) of untreated and cisplatin-treated TC-1 tumor-bearing mice in bar graphs. Data shown is representative for two individual experiments. Each dot represents data from an individual mouse. Data is expressed as the mean with SEM. Significance was determined by a Mann Whitney test. \*, *P*<0.05, \*\*, *P*<0.01, \*\*\*, *P*<0.001. D and E) Expression of CD70, CD80 and CD86 on TC-1 and C3 tumor cells. Upper panels: Representative flow cytometry plots for CD70, CD80 and CD86 on TC-1 and C3 tumor cells *in vitro*. TC-1 or C3 tumor cells (7000 cells/well) were cultured in a 96-well culture flat-bottom plate and after 48 hours incubation, cells were stained for CD70, CD80 and CD86 (solid grey line) or kept unstained (black dashed line). The expression of CD70, CD80 and CD86 was measured by flow cytometry. Lower panels: Expression of CD70, CD80 and CD86 on TC-1 and C3 from tumor-bearing mice. C57BL/6 mice were injected s.c with 1 × 105 TC-1 or 5 × 105 C3 tumor cells in the flank. On day 14 (TC-1 model) or day 20 (C3 model), mice were treated systemically with 10 mg/kg cisplatin. Four days later, tumors were dissected and analysed by flow cytometry. Shown is the expression of CD70 (left), CD80 (middle) and CD86 (right) on TC-1 and C3 tumor cells (7AAD-CD45-). Each circle represents data from an individual mouse. Mean with SEM is indicated. Significance was determined by a Mann Whitney test. \*, *P*<0.05, \*\*, *P*<0.01.

**Supplementary Materials and Methods**

**Cell line culture conditions**

Iscove's Modified Dulbecco's Media (IMDM) (BioWhittaker) supplemented with 8% fetal calf serum (FCS) (Greiner), 2 mM L-glutamine (Life Technologies), 50 IU/ml penicillin (Life Technologies) and 50 µg/ml streptomycin (Life Technologies) was used to culture tumor cell lines. Cells were cultured in a humidified incubator at 37°C and 5% CO2. D1 cells are long-term growth factor-dependent immature splenic DCs derived from C57BL/6 mice cells (1). *Mycoplasma* tests that were frequently performed for all cell lines by PCR were negative.

**Flow cytometric analysis of splenic and intratumoral immune cells**

For analysis of (tumor-infiltrating) immune populations, wild-type C57BL/6 mice were injected s.c with 1 × 105 TC-1 or 5 × 105 C3 tumor cells in the flank. On day 14 (TC-1 model) or day 20 (C3 model), mice were treated systemically with 10 mg/kg cisplatin. Four days later, lymphoid organs and tumors were collected after transcardial perfusion. Tumors were disrupted in small pieces and incubated with Liberase (Roche) in IMDM for 15 minutes at 37˚C. Spleen and lymph nodes were digested by incubating with 0.02 mg/ml DNAse and 1 mg/ml collagenase for 10 min at room temperature. Single-cell suspensions were prepared by mincing spleen and tumor pieces through a 70 µm cell strainer (BD Biosciences). Cells were resuspended in staining buffer (PBS + 2% FCS + 0.05% sodium azide) and incubated with various fluorescently labelled antibodies against: CD11b (clone M1/70), CD11c (clone N418), CD45.2 (clone 104), CD70 (clone FR70), CD80 (clone 16-10A1), CD86 (clone GL-1), 4-1BBL (clone TKS-1), OX40L (clone RM134L), ICOSL (clone HK5.3), F4/80 (clone BM8), Ly6C (clone HK1.4), Ly6G (clone 1A8), and CD90.2/Thy1.2 (clone 53-2.1). Antibodies were obtained from eBioscience and Biolegend. For dead cell exclusion, 7-aminoactinomycin D (7-AAD; Invitrogen) was used. Samples were analysed with a BD LSRII or LSRFortessa flow cytometer, and results were analysed using FlowJo software (Tree Star).

For intracellular cytokine staining,single cell suspensions of spleens isolated from naive and cisplatin treated mice were plated at concentrations of 8 × 105 cells in 96-well cell culture flat-bottom plates in the presence or absence of irradiated tumor cells and brefeldin A. After overnight incubation, cell surface markers were stained, and cells were fixed in 1% paraformaldehyde for 30 minutes. Thereafter, cells were washed, stained for cytokines and subsequently analysed by flow cytometry.

**Blood counts**

Complete blood counts were measured by the Sysmex XP-300 hematology analyser.

**Generation of bone marrow derived dendritic cells (BMDCs)**

Bone marrow derived dendritic cells (BMDCs) were generated from bone marrow of mice as follows. The femurs and tibias were collected and flushed. After red blood cell lysis, the bone marrow cells were cultured in a humidified incubator at 37°C and 5 % CO2 at a density of 40 × 106 cells/ml in IMDM medium containing FCS and 200 ng/ml murine recombinant Flt3L (generated in-house). After 8-10 days, cells were harvested from the cultures and live cells were enriched by high-density solution of Ficoll and centrifugation.

***In vivo* antibody usage and immune cell depletion**

The CD8 T-cell depleting monoclonal antibody clone 2.43 was purified from hybridoma cultures and 50 µg per mouse was administrated one day before cisplatin treatment. CD8 T-cell depletion was repeated every week and checked by staining for CD3 and CD8 followed by flow cytometric analysis. Depletion with 2.43 did not lead to depletion of CD8+ DCs (Supplementary Fig. 1C and 1D). CD4 and NK cell depletion was accomplished through injection of 50 µg per mouse GK1.5 (anti-CD4) and 100 µg per mouse PK136 (anti-NK1.1), respectively. CD4 and NK depletion was initiated one day before cisplatin treatment and repeated every 4-6 days. Agonistic CD27 antibody (clone: RM27-3E5) (25) was provided 4 times at 100 µg per mouse every 3 days. CTLA-4 blocking antibody (clone 9H10) was given 3 times at 200 µg per mouse every 3 days. All antibodies were administrated by intraperitoneal (i.p.) injection. Macrophage and dendritic cell depletion was achieved by i.p. administration of 1.5 mg/mouse clodronate liposomes 1 day before cisplatin treatment and repeated every 4-5 days (26).

**In vivo HMGB1 neutralization**

To neutralize HMGB1, chicken anti-HMGB1 polyclonal antibody was purchased from IBL International GmbH (Manufactured and sold by SHINO-TEST Corporation, Japan) and was used at the concentration of 2 mg/kg to neutralize HMGB1 in mice as suggested by the manufacturer.

**Toxicity measurements**

To measure toxicity induced by cisplatin, serum samples of mice were taken 4 days after cisplatin treatment, and the levels of aspartate transaminase (AST) and alanine transaminase (ALT) were measured according to standard protocols of the Department of Clinical Chemistry of LUMC.

**Anti-tumor vaccination**

Concurrent with the first administration of cisplatin on day 8, mice were vaccinated in the contralateral flank with synthetic long HPV16 E743-77 peptide (GQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIR) dissolved in 200 µl PBS and emulsified with Montanide ISA51 (2). Cisplatin was obtained from Pharmachemie B.V. (Haarlem, The Netherlands) and Accord Healthcare LTD (Middlesex, England).

**T-cell activation measurement**

For T-cell proliferation determination, TCR transgenic pmel-1 CD8+ T cells were isolated from spleens and lymph nodes by using the mouse CD8+ T lymphocyte enrichment set (BD Biosciences). CD8+ T cells were labelled with CFSE by incubation in PBS containing 1 mM CFSE and 0.1% BSA at 37°C for 10 minutes. Cells were then washed three times with PBS containing 0.1% BSA. TC-1 cells (7000 cells/well) were cultured in a 96-well culture flat-bottom plate (Corning Costar) in the presence of 1.0 µg/m cisplatin. After overnight incubation, cells were washed and further cultured with fresh medium. After another overnight incubation, D1 cells or BMDCs (5 × 104 cells/well) were co-cultured with the CFSE-labelled pmel-1 cells (0.5 × 106/well) in the presence of long mgp100 EGP20-49 for 72 hours at 37°C, 5% CO2. Then, cells were harvested and stained for Thy 1.1 (CD90.1). After washing, the amount of CFSE dilution was measured by flow cytometry. To measure the functionality of T cells, IFN-γ was measured in the supernatant of cells by ELISA. To determine the functionality of T cells in the presence of cisplatin-treated tumor cells and different BMDCs, TC-1 tumor cells were treated with 1.0 µg/ml cisplatin overnight or left untreated, and BMDCs derived from wild-type, *Cd70-/-*, *Cd80/86-/-* and *Cd70/80/86-/-* mice were added and co-cultured with CD8+ pmel-1 T cells in the presence of gp10025–33 peptide. After 72 hours incubation, supernatant was collected and IFN-γ was measured by ELISA. IFN-γ production is normalized compared to stimulation with untreated tumor cells

**Analysis of costimulatory molecule expression *in vitro***

To dissect the effect of cisplatin-treated tumors on maturation of dendritic cells, TC-1 tumor cells were harvested and seeded at 7000 cells per well in a 96-well cell culture flat-bottom plate. Six hours later, cisplatin was added and the cells were incubated overnight. The next day, cells were washed and 5 × 104 D1 cells were added to the cells. To analyse the effect of HMGB1 and type I IFN on DCs, 1 × 104 wild-type BMDCs or 5 × 104 D1 cells were incubated with 20 ng/ml HMGB1 protein (IBL international) or 1.3 µg/ml type I IFN (produced as described ((3)). To block IFNAR, InVivoMAb anti-mouse IFNAR-1 (Clone: MAR1-5A3, BioXcell) was used. LPS (1 µg/ml) (Sigma-Aldrich) and anti-CD40 antibody (1 µg/ml) (clone HM40-3, BD Biosciences) were used as positive control. All samples were incubated for 72 hours at 37˚C. Cells were then harvested and stained with fluorescently labelled antibodies against costimulatory molecules (CD70, CD80, CD86, 4-1BBL, OX40L, ICOSL) after which the samples were analysed by flow cytometry.

**Intracellular cytokine staining after 4 days *in vitro* stimulation**

Spleens were dissected and single cell suspensions seeded at about 6 × 106 cells per well in a 24-well cell culture flat-bottom plate and stimulated for 4 days with irradiated TC-1 or MCA-205 tumor cells or left unstimulated. After four days, cells were harvested and live cells were enriched by high-density solution of Ficoll and centrifugation. Cells were rested for 2-3 days in the presence of 10 IU IL-2 per ml. After resting, harvested cells were transferred to 96-well culture round-bottom plate and stimulated overnight as described above in presence of Brefeldin A. Next, cells were stained for intracellular IFN-γ and TNF production.

**MTT assay**

TC-1 tumor cells (7000 cells per well) were cultured in a 96-well culture flat-bottom plate for 24 hours. Following overnight treatment with escalating dosages of cisplatin, cells were extensively washed and grown for an additional 24 hours in fresh medium. Cell viability assay was determined using a standard colorimetric MTT (3-4,5-dimethylthiazol-2-yl-2, 5-diphenyl-tetrazolium bromide) reduction assay. Absorbance was measured with at a test wavelength of 570 nm, and a reference wavelength of 655 nm.

**Multiplex assay**

TC-1 tumor cells were incubated with indicated amounts of cisplatin. After overnight incubation, cells were washed and incubated overnight. Then, supernatant was collected and stored at −80°C until further use. Cytokines were measured using a mouse Bio-Plex Pro Mouse Cytokine 23-plex immunoassay (Bio-Rad, Herculus, CA, United States) according to manufacturer's protocol. This multiplex assay detects: Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3 IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17a, KC, MCP-1 (CCL2), MIP-1α, MIP-1β, RANTES, TNFα. IFNα was measured with a mouse ProcartaPlex multiplex immunoassay (eBioscience).

**HMGB1 ELISA**

To measure HMGB1 produced by cisplatin-treated tumor cells, TC-1 tumor cells were incubated with indicated amounts of cisplatin. After overnight incubation, cells were washed and incubated overnight, and HMGB1 levels in the supernatant were determined by ELISA (IBL international) according to the manufacture’s protocol.

**Proteome analysis of chemotherapy-treated tumor cells by mass spectrometry**

To determine the proteins released after chemotherapy treatment, TC-1 tumor cells were cultured at concentration of 4 × 106 in T175 CELLSTAR® Tissue Cell Culture Flasks (Corning). Six hours later, cells were treated with 1 or 2.5 µg/ml cisplatin or kept untreated for overnight. Next, the cells were extensively washed and the medium is replaced with fresh medium without FCS. After overnight incubation, the supernatants were collected and centrifuged in two steps; first at 1500 rpm for 15 min and then at 2,500 rpm for 15 min both at 4°C to remove cells and cell debris. Cell-free supernatants were acidified to 1% formic acid, concentrated 20× by lyophilization and filter fractionated using 10 kDa Microcon filters (Millipore). The >10 kDa fraction was subjected to a modified filter aided sample preparation procedure (4). After two 25 mM NH4HCO3 washes, the proteins were reduced for 30 min at 37°C on the filter with 200 μl 5 mM DTT and thereafter alkylated for 30 min at room temperature by the addition of 200 μl of 30 mM iodoacetamide. Next, proteins were washed twice with 25 mM NH4HCO3 and digested overnight at 37 °C with 5 μg of trypsin. Tryptic peptides were recovered from the filtrate and analysed by mass spectrometry without further purification. Samples were analysed by online nano high-performance liquid chromatography MS/MS.

Tryptic peptides were analysed using an Easy nLC1000 (Thermo, Bremen, Germany) coupled to a Q-Exactive mass spectrometer (Thermo). Injection was done onto a homemade precolumn (100 μm × 15 mm; Reprosil-Pur C18-AQ 3 μm, Dr. Maisch, Ammerbuch, Germany) and elution via a homemade analytical column (15 cm × 50 μm; Reprosil-Pur C18-AQ 3um). The gradient was 0% to 30% solvent B (90% ACN/0.1% FA) in 120 min. The analytical column was drawn to a tip of ∼5 μm and acted as the electrospray needle of the MS source. The Q-Exactive mass spectrometer was operated in top10-mode. Parameters were as follows: full scan, 70,000 resolution, 3,000,000 AGC target, max fill time 20 ms; MS/MS, 35,000 resolution, 100,000 AGC target, 60 ms max fill time, 17,400 intensity threshold. Apex trigger was set to 1–5 s, and allowed charges were 2–5. Proteome Discoverer version 2.1 was used for peptide and protein identification, using the mascot node for identification, using mascot version 2.2.04 with the Uniprot/SwissProt database (June 2015; 548.586 entries). Up to two missed cleavages were allowed, and methionine oxidation was set as a variable modification; carbamidomethyl on Cys was set as a fixed modification. Peptide assignments were made with a tolerance of 10 ppm. MS/MS fragment tolerance was 20 mmu. Protein identifications were assigned on the basis of a minimum of two confident peptides at 1% false discovery rate (FDR) using the Proteome Discoverer target decoy PSM validator at a strict FDR of 1%. For protein quantitation emPAI values were calculated on the basis of the top 3 intense peptides of each protein (5).

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