Supplementary Methods

NCOA1 Directly Targets M-CSF1 Expression to Promote Breast Cancer Metastasis

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Generation of Tg(NCOA1) mice. The hNCOA1 cDNA was cloned into a pBluescript II SK-based vector between an Asc I and a Sva I restriction enzyme sites. This vector contains the MMTV-LTR promoter that drives the hNCOA1 transgene expression in the mammary epithelial cells (MECs) and a bovine growth hormone Poly-A (bGHpA) signaling sequence for stabilizing the transgene mRNA. The full-length transgene (Fig. 1A) was isolated from the vector backbone by Not I digestion and microinjected into fertilized 0.5-day oocytes collected from FVB female mice. The injected oocytes were implanted into the oviducts of foster mothers and the embryos were allowed to develop to term as described (1). Transgenic founders were identified from genomic DNA extracted from mouse ear tips by PCR using a pair of transgene-specific primers (Supplementary Table S1). Individual Tg(NCOA1) mouse lines were established by crossing transgenic founders with wild type (WT) FVB mice. The transgene expression was assayed by real time RT-PCR using hNCOA1-specific primers (Supplementary Table S1) and by immunohistochemistry (IHC) using a NCOA1 antibody.

Immunohistochemical analysis of NCOA1 and CSF1 with human breast tumor tissue microarrays. Breast tumor samples (n=560) from archival cases during 1987 to 1999 at St Vincent’s University Hospital were collected from primary surgeries before endocrine therapy. After surgery, patients received either 20 mg/day of tamoxifen (n=360) or no endocrine treatment (n=200) for five years, which was discontinued only in those patients who suffered a relapse while on endocrine therapy. Excluded from the analysis were patients who did not have breast surgery, those who had neoadjuvant therapy or those whose tissue specimens were irretrievable. Data on the patients included pathological characteristics (tumor size, grade, lymph node status, recurrence, estrogen receptor status) and treatment with radiotherapy, chemotherapy or tamoxifen. Archival tissues were used to construct tissue microarrays (TMAs) and prepare sections (2, 3). For IHC, rehydrated sections were microwaved in 0.01 M sodium citrate for 15 minutes and immersed in 3% H2O2 for 10 minutes. After blocking in 10% horse serum for 1 hour, sections were incubated with anti-NCOA1 (#2191, Cell Signaling, Danvers, MA) or anti-CSF1 (ab52864, Abcam, Cambridge, MA) antibody overnight at 4°C. After incubating with appropriate biotin-labeled secondary antibodies and peroxidase-conjugated avidin (Vector Laboratories, Burlingame, CA), positive signals were developed in the 3,3-diaminobenzidine tetrahydrochloride solution. Sections were counterstained with hematoxylin.

Examination of mammary gland (MG) morphology, epithelial proliferation, MG tumor growth and lung metastasis. The fourth pairs of MGs were collected from female littermates of wild type (WT) and Tg(NCOA1) mice at specific ages and fixed in Carnoy’s fixative for whole mount staining or in 4% paraformaldehyde for paraffin section as described (4-6). Five mice were examined for each group. The growth index of the inguinal MG was measured by the ratio of the length from the lymph node to the leading edge of the ductal tree to the length of the fat pad. The proliferative cells in the MG epithelium were detected by immunohistochemistry using Ki67 antibody on MG sections as described (6, 7). The percentage of proliferating cells was obtained by counting the number of Ki67-positive epithelial cells against the total number of epithelial cells in ductal cross sections. RCAS-PyMT (polyoma middle T antigen) viruses were produced and injected into the mammary ducts of Tg(TVA) and Tg(NCOA1)×Tg(TVA) mice at 10 weeks of age as described (8). MG tumor development was examined by palpation starting at two weeks after viral introduction. For Tg(Neu) and Tg(NCOA1)×Tg(Neu) mice, palpation examination of MG tumors began at 5 months of age. Tumor initiation and tumor volume data were collected from these mice as described (9). Two hundred µl of blood was collected from the right ventricle of each mouse harboring MG tumors for 9 weeks. These blood samples were cultured to determine cancer cell colony-forming units as described (9). Lungs were also collected from these mice for histological examination of metastasis as described (9). The index and frequency of metastases were also calculated as described (9).

Immunohistochemistry, Western blotting and ELISA. Immunohistochemistry was performed on de-paraffinized MG and tumor sections using antibodies against NCOA1 (Cell signaling), Ki67 (Abcam), CSF1 (Abcam) and F4/80 (Abcam) as described (10). MG tumor tissue lysates with 50 µg of total protein in each were analyzed by Western blotting using some of the above antibodies. Western blotting using β-actin antibody (Sigma) served as a loading control. For ELISA, PyMT×Ncoa1-W1/W2 and PyMT×Ncoa1-K1/K2 MG tumor cells were developed from primary tumors as described (10, 11) and cultured in 6-well plates to 90% confluence. These cells were starved in serum-free DMEM medium for 2 days. CSF1 concentration in the conditioned medium was measured using the Quantikine Mouse M-CSF ELISA Kit (R&D
Systems) and normalized to the total cellular protein amount in each well as described (9).

**Quantitative real time RT-PCR (qPCR).** qPCR was performed as described (10, 11). Briefly, total RNA was extracted from tumor tissues or cells using TRIZOL reagent (Invitrogen). Reverse transcription and real time PCR were performed using matched Universal Taqman Probes (Roche) and mRNA-specific primers listed in Supplementary Table S1. The 18S rRNA was measured as an endogenous control.

**Knockdown and expression of NCOA1.** NCOA1 or CSF1 mRNA was knocked down by synthetic siRNA (Dharmacon) or lentivirus-mediated shRNA expression (Baylor College of Medicine) in PyMT×Ncoa1-W1/W2 mouse MG tumor cells or in human MCF-7, MDA-MB-231 or MDA-231-LM3.3 cells. Scrambled siRNA or non-targeting shRNAs were used as controls as described (10, 11). Adenovirus-mediated NCOA1 or GFP (green fluorescence protein) expression in PyMT×Ncoa1-K1/K2 mouse MG tumor cells or MDA-MB-231 cells was carried out as described (10, 11). qPCR was performed to examine the efficiency of these manipulations.

**Chromatin immunoprecipitation (ChIP) assay.** ChIP assays were performed with crosslinked DNA-protein complexes extracted from MDA-MB-231 cells, using 2 μg of NCOA1 or c-Fos antibodies, and the protein-G/A beads as described (10, 11). Six pairs of specific primers (Supplementary Table S1) were designed to amplify different regions of the human CSF1 promoter by PCR.

**References**