Supplementary Methods:

Microarray Data analysis. For unsupervised analysis, genes were filtered using the following criteria: good quality spot, spot intensity greater than twice background on at least 70% of the arrays, and a two-fold or greater increase in expression over median on at least two arrays. Using these criteria, 1,970 genes passed filtering and were analyzed by hierarchal clustering as described below. For supervised analysis, genes that were significantly up- or down-regulated and which correlated with a variable of interest (e.g., RAS/RAF mutation status, described further below) were identified using Significance Analysis of Microarrays [SAM; (1)]. For the SAM analysis, data were excluded for genes with poor spot quality or with a mean intensity greater than twice the median background for both the red and green channel in at least 90% of the experiments (21,911 genes passed filtering). The $\log_2$ of the median red intensity over median green intensity was calculated for each gene. Missing data were imputed using SAM with 100 permutations and *k*-nearest neighbors (KNN) with $k = 10$. Delta values were adjusted to obtain the largest gene list with a false discovery rate (FDR) < 5%. SAM was used to analyze both discrete (e.g., RAS mutation status) and continuous (e.g., % G1 arrest after IR) variables. Once supervised and unsupervised gene lists were identified, hierarchical clustering analysis was conducted using the program Cluster (http://rana.lbl.gov/EisenSoftware.htm) to perform median centered, average-linkage clustering. Clusters were visualized using Treeview (2, 3). All data shown in the heat map figures are median-centered.

Tissue Microarray Construction. Tissue sections from each block were stained with hematoxylin and eosin (H&E) and reviewed by a pathologist to define the selective areas to be punched. To preserve the original tissue block, either 0.6 mm cylindrical cores of tissue were punched, then inserted in a standard 4.5 x 2 x 1 cm recipient block using a Tissue Microarrayer (Beecher Instruments, Silver Spring, MD, USA) with an edge-to-edge distance of 0.1 or 0.15 mm. 570 samples of melanomas representing all clinical stages were then cut to 5 µm sections

and placed on glass slides using an adhesive tape transfer system (Instumedics, Inc., Hackensack, NJ) with UV cross-linking. The arrays were constructed and analyzed at two-fold redundancy.

Immunofluorescent Detection for AQUA Analysis. The tissue microarray slide was stained as described previously (4, 5). In brief, the slides were deparaffinized by rinsing with xylene, followed by two changes of 100% ethanol and two changes of 95% ethanol. Antigen retrieval was performed in a pressure cooker containing 6.5 mM citrate (pH 6.0) and endogenous peroxidase activity was blocked with 2.5% hydrogen peroxide in methanol for 30 min at room temperature. The slide was washed with Tris-buffered saline (TBS), incubated in 0.3% bovine serum albumin (BSA)/1X and TBS for 30 min at room temperature to reduce nonspecific background, and then stained with a combination of rabbit primary antibody and mouse monoclonal antibody for the S100 protein expressed in essentially all melanomas (AM058, Biogenex) diluted in BSA/TBS at 4°C overnight. The primary antibodies in this study were used as follows: E-cadherin (Transduction Labs), 1:400; P-cadherin (Transduction Labs), 1:250; N-cadherin, (Zymed), 1:150; CD24 (Neomarkers), 1:50; and cytokeratin 18 (Neomarkers), 1:100. The secondary antibodies, Alexa 546-conjugated goat antirabbit (1:200, Molecular Probes) plus Envision anti-rabbit (neat; DAKO) diluted in BSA/TBS were applied for one h at room temperature. 4', 6-Diamidino-2-phenylindole (DAPI) was included with the secondary antibodies to visualize nuclei. The slide was washed with BSA/TBS (three times for 5 min) and then incubated with Cy5-tyramide (Perkin-Elmer Life Science Products) and activated by horseradish peroxidase, resulting in the deposition of numerous covalently associated Cy5 dyes immediately adjacent to the horseradish peroxidase-conjugated secondary antibody. Cy5 was used because its emission peak (red) is well outside of the green-orange spectrum of tissue autofluorescence. The slides were sealed with coverslips with an antifade-containing mounting medium (with 0.6% n-propyl gallate).

Automated Image Acquisition and Analysis. The AQUA automated image acquisition and analysis was performed as described previously (4). Briefly, images of the tissue microarray were captured through an Olympus BX51 microscope with automated x, y, and z stage movement with an Olympus Motorized Reflected Fluorescence System and software (IP lab v3.60, Scanalytics, Inc.) equipped with Cooke Sensicam QE High Performance camera. Areas of tumor were distinguished from stroma and non-melanoma tissue by creating a mask with the S100 signal tagged with Alexa 546. Expression of S100 protein was used to identify the tumor mask and 4', 6-diamidino-2-phenylindole (DAPI) was used to identify the nuclear compartment. Areas of the tumor mask without DAPI were considered 'nonnuclear' or 'cytoplasmic'. The target markers were visualized with Cy5 (red). Rows and columns of the histospots were then identified, missing histospots filled in, allowing each histospot to be identified by its coordinates, and recorded based on its position in the grid. Subsequently, monochromatic, high-resolution (1024 x 1024 pixel, 0.5µm resolution) images were obtained of each histospot, both in the plane of focus, and 8 µm below it, and recorded in an image stack as bitmaps. A resolution of 0.5 µm is suitable for distinguishing between large subcellular compartments such as the cell membrane/cytoplasm and nuclei. The targets were measured in both cytoplasmic and nuclear compartments within the S100 tumor mask on a scale of 0 to 4095 and expressed as target signal intensity relative to the respective cytoplasmic or nuclear compartment area.

References

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