Supplemental Methods and Materials

**TNC TMA immunohistochemistry**

Melanoma tissue microarrays ME1004 and ME242 were purchased from Biomax (Rockville, MD) and stained with anti-TNC antibody that recognizes the EGFL repeats (ab6393, Abcam, Cambridge, MA) as previously described (Hood et al. 2010). Images were acquired with an Olympus 40X 0.75 NA objective on Olympus BX-40 microscope.

**Immunoblotting and immunostaining**

Immunoblotting and immunostaining were performed as previously described (Hood et al. 2010). Antibodies used were: anti-TNC recognizing FN repeats (MAB33581, R&D Systems), anti-TNC recognizing EGFL repeats (MAB2138, R&D Systems), anti-pMLC2 Ser-19, anti-pMLC2 Thr-18/Ser-19, anti pMYPT Thr-853 (#3675, #3674 and #4563, respectively, Cell Signaling Technology) and anti-GAPDH (G9545, Sigma-Aldrich). All secondary antibodies were from Sigma-Aldrich or Molecular Probes, Invitrogen. Actin fibers were stained with 488- or 568-Alexa Fluor phalloidin (Invitrogen) and nuclei with DAPI. Images were acquired with an Olympus 60X 1.25 NA or Olympus 20x 0.4 NA objective on Olympus BX-40 microscope.

**Generation of TNCEGFL and TNCFULL constructs and TNCEGFL expressing melanoma cell lines**

Full length TNC construct (X78565) was obtained form Dr. Harold Erickson (Duke University, Durham, NC)(Aukhil et al. 1993). The first half of the TNC sequence was amplified with introduced NotI and EcoRV cutting sites, of total length of 2355 bases, and cloned into pcDNA3.1(-) vector (Invitrogen). The primers for amplification were 5’-ATA CTC AAG CGG CC G CAT GGG GGC CAT GAC TCA-3’ and 5’-CGG ATA TCT CAT ACT CTT GCC CAG GA-3’. Subsequently, TAA stop codon was introduced instead of TCA codon at the end of 14th EGFL repeat with Quick Change II Site Directed Mutagenesis Kit (Stratagene) and primers 5’-GGG AGA CAC CTCTTA GCA GTC TTC TCC G-3’ and 5’-CGG AGA AGA CTG CTA AGA GGT GTC TCC TCC C-3’. WM983A cells were transfected with TNCEGFL using Lipofectamine (Invitrogen) and G418 resistant clones were selected. Clone expressing the highest amount of TNCEGFL (C1) was selected for further analysis and clone with lower level of TNCEGFL expression (C3) for comparison in some assays. As a control, a
monoclonal WM983A isolate expressing empty pcDNA3.1(-) vector was established. For the TNCFULL construct the full length TNC was cloned into the same vector and G418 resistant clones were selected. The same vectors EV, TNCEGFL and TNCFULL were used for transient transfection in WM35 cell line.

**Cell spreading and inverted centrifugation assays**

For spreading assays cells were trypsinized and re-plated on plastic culture dishes in regular medium or in the presence of 5µM Y27632 (Calbiochem). After 2, 4, 6 and 8 hours images were acquired using Olympus IX70 microscope with a 10X 0.3 NA objective, cells were counted (n=100 per treatment) and percentage of spread cells was determined. Completely round phase-bright cells were counted as non-spread and those that lacked round shape and had membrane protrusions were counted as spread. Data was collected from three independent experiments.

Inverted centrifugation assay was performed as described (Shao et al. 2010). In this assay, after 2, 4, 6 and 8 hours, we compared stationary plates washed with PBS against plates spun inverted at 1000 rpm (150g). This was used to calculate the % of attached cells compared to not centrifuged plate controls.

**2D migration assays and cell tracking**

For wound healing assay cells were seeded in regular medium and let attach 12h and then quiesed in 0.5% FBS medium for 24h. The cross wound was introduced with a yellow pipette tip, and the cross was used as a reference of position for taking images. If used, 5µM Y27632 (Calbiochem) was added at the time of introducing the wound. Images were taken at 0 and 22h post scratching using Olympus IX70 microscope with a 4X 0.13 NA objective. Area of the wound was calculated using ImageJ software and results were expressed as % of the area closed within 22h.

For imaging of single cell movement of WM35, WM983A and WM983B cells, plates were coated with 2µg/cm² of rat tail Collagen I (BD Biosciences, Bedford, MA) or 2µg/cm² of Collagen I and 2µg/cm² of human Tenascin C (Millipore, Temecula, CA) in PBS for 2 hours at ambient temperature and then blocked for 30 minutes with 0.1% BSA in PBS. 2x10⁴ cells per well were seeded and let attach for 12 hours. Cells were labeled with Cell Tracker red CMPTX (Invitrogen, Carlsbad, CA) and after 6h in 0.5% FBS medium imaged for 8 hours every 10 minutes in TRITC and phase channels with Nikon 10X 0.5 NA objective on Nikon Ti-E inverted microscope with CO₂ stage incubator
Cell migration speed and track length and displacement were analyzed with Imaris software (Bitplane, Zurich, Switzerland) based on tracking of movement of fluorescence as autoregressive motion. Experiment was repeated two times and total of more than 50 tracks per treatment were analyzed.

For tracking of WM35 cell movement upon expression of TNC constructs, cells were transfected with EV, TNCFULL or TNCEGFL, and 24h later imaged for 8h every 10 minutes.

For tracking of WM983A-EV and WM983A-TNCEGFL cell movement, cells were seeded on glass-bottom dishes (MatTek Corporation, Ashland, MA) and let grow for 72 h before imaging to allow deposition of the matrix and then imaged and analyzed as described above.

3D invasion assay

Matrigel invasion assay was performed in BD BioCoat Matrigel Invasion Chamber per manufacturer instructions (354480, BD Biosciences, Bedford, MA). Cells were let to invade for 48h or 72h as specified in the legends. The invaded cells at the bottom side of membrane were stained with DAPI and five random 10x fields were counted, with each condition in triplicate. Experiments were repeated three times and average number of invaded cells was expressed as number of invaded cell per 10x field or relative to WM983A-EV.

Confocal imaging of Matrigel invading cells

Cells were seeded as for Matrigel invasion assay and let invade 48h. Matrigels were stained as described with modifications (Hooper et al. 2006). Briefly, Matrigel inserts were washed with PBS, fixed 30 minutes in 4% formaldehyde 0.25% glutaraldehyde in PBS, treated with 0.2% Triton X for 30 min on ice, and stained with 0.2µg/ml 568-Alexa-Fluor phalloidin (Molecular Probes, Invitrogen) and 2µg/ml DAPI (Sigma) in dark for 3h. After washing with PBS samples were incubated with anti-TNC or anti-ppMLC antibody for 12h at 4°C and another 2h at ambient temperature with anti-rat 488-Alexa Fluor secondary antibody (Invitrogen). Membranes were cut out with a razor blade and mounted on glass slides in PBS. Images were taken on Nikon Sweptfield Confocal Microscope (TSI inverted) using 10X and 60X 1.4 NA objectives. 3D volume representations of Z-stacks were made using Nikon Elements software.
**Skin organ cultures and H&E staining**

Skin organ cultures were established as previously described (Simpson et al. 2010) with modifications. Briefly, 5x10^5 primary human fibroblasts were seeded in collagen gels, and after 5 days in submerged culture 1x10^6 normal human keratinocytes were seeded on top. The next day 1x10^5 WM983A-EV or WM983A-TNCEGFL cells were seeded in the center of the plug within the cloning rings, let attach for 8h and then the cloning rings were removed. After 3 days, the cultures were lifted to air liquid interface to allow epidermis maturation and medium was replenished every other day for total of 20 days of culture. The samples were fixed and paraffin embedded and H&E stained as previously described (Hood et al. 2010). Experiment was repeated two times with similar results.

**Gelatin zymography**

Gelatinase activities of MMP2 and MMP9 in WM983A-EV and WM983A-TNCEGFL conditioned medium and lysates were assessed according to prior protocols (Toth et al. 2001).

**PCR array**

RT^2 Profiler PCR Array of Human Extracellular Matrix and Adhesion Molecules (PAHS-013A, SABiosciences, Frederick, MD) was performed per manufacturer instructions in 96 well format with 1µg of total RNA. Experiment was repeated two times and data was analyzed with SABiosciences PCR Array analysis web-based Utility (http://sabiosciences.com/pcrarraydataanalysis.php).

**Proximity ligation assay (PLA)**

WM983A-EV and WM983A-TNCEGFL cells were grown on glass cover slips, fixed with 4% formaldehyde, treated with 0.2% Triton-X, blocked with 5% goat serum and incubated with primary rabbit anti-EGFR (#2232, Cell Signaling Technology) and mouse anti-TNC (ab6393, Abcam) that recognizes EGFL overnight at 4°C. PLA was performed according to the manufacturer’s instructions (Duolink II Red Starter kit, Olink Bioscience, Uppsala Sweden). Briefly, rabbit and mouse specific oligonucleotide conjugated secondary antibodies (PLA probes) bind to the primary antibodies. Next, probes are annealed if they are in close proximity (<4 0nm) and in the presence of fluorescently labeled onligonucleotides allow rolling circle amplification. The signal
from the close physical association between the proteins of interest is visualized as red dots. Nuclei were labeled with DAPI and images were taken on Nikon Sweptfield Confocal Microscope (TSI inverted) using 40 1.2 NA objective. 20 Z plane images were taken every 1µm and the intensity and number of red signals were analyzed with Image J Particle Analyzer for at least 20 cells in each treatment, and expressed as average integrated density per cell. Experiment was repeated two times. Controls with single anti EGFR and anti TNC antibodies were performed and they gave low or no signal.

**Statistical testing**

All statistics of quantification in this study, including spreading assay, wound healing assay, Matrigel invasion assay, RT-PCR and live cell tracking experiments were performed by two-tailed Student's t test with a P < 0.05 being required to be considered significant and shown as mean ± SD or SEM (if each experiment was performed in triplicate).

**Supplemental References**

Aukhil, I., P. Joshi, Y. Yan, et al. (1993). "Cell- and heparin-binding domains of the hexabrachion arm identified by tenascin expression proteins." J Biol Chem 268(4): 2542-2553.

Hood, B. L., J. Grahovac, M. S. Flint, et al. (2010). "Proteomic analysis of laser microdissected melanoma cells from skin organ cultures." J Proteome Res 9(7): 3656-3663.

Hooper, S., J. F. Marshall and E. Sahai (2006). "Tumor cell migration in three dimensions." Methods Enzymol 406: 625-643.

Shao, H., J. H. Wang, M. R. Pollak, et al. (2010). "alpha-actinin-4 is essential for maintaining the spreading, motility and contractility of fibroblasts." PLoS One 5(11): e13921.

Simpson, C. L., S. Kojima and S. Getsios (2010). "RNA interference in keratinocytes and an organotypic model of human epidermis." Methods Mol Biol 585: 127-146.

Toth, M. and R. Fridman (2001). "Assessment of Gelatinases (MMP-2 and MMP-9 by Gelatin Zymography." Methods Mol Med 57: 163-174.
Figure S1. Expression of TNCFULL or TNCEGFL impairs WM35 cell migration. (a) Immunoblotting of TNC under non-reducing conditions with the antibody recognizing EGFL repeats (R&D Systems) and ppMLC: whole cell lysates and matrix at the end of live cell tracking experiment; increased ppMLC in cells transfected with TNC constructs. Intensities of protein bands were determined by integrating optical density over the band area using Image J software, normalized to the GAPDH levels and divided by the lane 1. (b) Individual cell speed and track straightness of WM35 cells transfected with TNCFULL or TNCEGFL are significantly decreased compared to WM35-EV cells. N > 50 tracks per phenotype. * p < 0.05, ** p < 0.01. TNCEGFL expressing cells have more severe phenotype than TNCFULL.
Figure S2. TNCEGFL expressing cells have anti adhesive phenotype that persists on fibronectin coated surfaces and have unchanged proliferation rates and MMP2 activity as WM983A-EV cells. (a) Quantification of cell attachment assay (left panel) and wound healing assay (right panel) on 2µg/cm² fibronectin coated surface. (b) Number of WM983A-EV and WM983A-TNCEGFL cells 48h post plating determined with trypan blue dye exclusion. (c) Gelatin zymogram of WM983A-EV and WM983A-TNCEGFL conditioned medium or cell lysates.

(a) Quantification of percent of attached cells over time.

(b) Comparison of cell attachment and wound closure between EV and TNCEGFL.

(c) Gelatin zymogram showing MMP2 activity.
Figure S3. Proximity ligation assay for physical association of EGFR and TNC. PLA assay was performed with primary anti EGFR antibody (Cell signaling) and anti TNC antibody that recognizes EGFL repeats (ab6393, Abcam). Close physical association of the two proteins is visualized as a red fluorescent signal (a) Representative maximum intensity projections from 20 planes 1 µm apart. PLA signal is red, nuclei stained with DAPI. (b) Average integrated density of the red signal per cell in each plane was calculated with Image J software Particle Analyzer. The distance of 20 represents the surface of the coverslip.
Supplemental table 1. Integrin profile of WM9893A-EV and WM983A-TNCEGFL cell lines determined by PCR Array

| gene symbol | WM983A-TNCEGFL Ct | WM983A-EV Ct | fold change |
|-------------|--------------------|--------------|-------------|
| ITGA1       | 25.60              | 26.23        | 1.34        |
| ITGA2       | 28.35              | 29.09        | 1.45        |
| ITGA3       | 25.85              | 25.87        | 0.88        |
| ITGA4       | 21.79              | 22.03        | 1.03        |
| ITGA5       | 25.92              | 25.40        | 0.61        |
| ITGA6       | 20.59              | 20.59        | 0.87        |
| ITGA7       | 30.17              | 30.58        | 1.16        |
| ITGA8       | 35.00              | 35.00        | 0.87        |
| ITGAL       | 34.60              | 34.81        | 1.01        |
| ITGAM       | 32.18              | 35.00        | 6.14        |
| ITGAV       | 22.20              | 21.86        | 0.67        |
| ITGB1       | 20.05              | 20.14        | 0.93        |
| ITGB2       | 33.03              | 35.00        | 3.41        |
| ITGB3       | 25.83              | 26.14        | 1.08        |
| ITGB4       | 34.74              | 34.18        | 0.59        |
| ITGB5       | 25.15              | 25.95        | 1.51        |

| gene symbol | WM983A-TNCEGFL Ct | WM983A-EV Ct | fold change |
|-------------|--------------------|--------------|-------------|
| HPRT1       | 22.04              | 21.83        |             |
| RPL13A      | 19.21              | 18.89        |             |
| GAPDH       | 17.24              | 16.99        |             |
| ACTB        | 14.33              | 15.34        |             |

* The grey shaded genes’ average threshold cycle is relatively high (> 30), meaning that its relative expression level is quite low and thus quantitation is difficult, in both control and test samples, and thus, fold changes while in two seemingly significant, are likely biologically irrelevant. All other changes for mRNA that are present at readily measurable levels are not statistically significant.