SUPPLEMENTARY TEXT S1

References to Figure 1b

miR-17/302: [1]
miR-19: [1-5]
miR-21: [6]
miR-22: [1, 7, 8]
miR-23: [9]
miR-25: [1]
miR-26: [10]
miR-136: [11]
miR-141: [12]
miR-144: [11]
miR-193: [9]
miR-214: [13]
miR-216: [14]
miR-217: [14]
miR-221: [15]
miR-486: [16]
miR-494: [17]

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SUPPLEMENTAL TEXT S2

Analysis CDKN2A deletion, PTEN deletion and BRAF and NRAS mutation in the human melanoma cell lines and tissues.

23 out of the 28 lines (82.1%) showed at least a partial deletion of CDKN2A (Figure 2a). Recent evidence indicates that there are two additional melanoma-related oncosuppressor genes located at 9p21, one centromeric (CDKN2B) and one telomeric (MTAP) to CDKN2A (Conway et al., 2010). If the partial or complete deletion of CDKN2B and MTAP loci is included, the total count of cell lines showing deletion at 9p21 locus raises to 89.3% (25 out of 28). The rate of CDKN2A partial or complete deletion alone in melanoma tissues is 37.2% (16 out of 43) (Figure 2b). The inclusion of CDKN2B and MTAP raises the count to 48.8% (21 out of 43). The higher rate shown by melanoma cell lines compared to melanoma tissues is possibly due to the fact that the loss of 9p21 genetic material is advantageous for growth in culture (Bennett, 2008). In agreement with previous findings (Indsto et al., 1998), a positive correlation between partial or complete deletion at the PTEN locus and partial or complete deletion at the CDKN2A locus was observed (Figure S1a).

PTEN was found deleted, either partially or completely, in 13 out of the 28 cell lines (46.4%) and in 23 out of the 43 tissues (53.5%) (Figure 2). In order to confirm the reliability of the genomic qPCR used, PTEN protein was detected by western blot (cell lines) and immunohistochemistry (IHC, tissues). The PTEN band was absent in the primary lines WM98.1 and WM853.2 and in the metastatic lines Sk-Mel-85 and Sk-Mel-94, consistent with the complete deletion of at least a portion of PTEN locus. In contrast, WM35 and SK-Mel-103 still showed some residual expression, consistent with the only partial deletion at the PTEN locus (Figure S2). IHC analysis revealed a wide decrease in PTEN protein levels in melanoma tissues: the vast majority of the samples (39 out of 43, 90.7%) did not contain more than 50% of positive cells (Figure 2b). We found that PTEN
partial or complete deletion and PTEN protein levels are inversely correlated (Figure S1b). This result confirms the reliability of our genomic qPCR approach. It also indicates that, although a role can be played by loss-of-function mutations and promoter methylation, which have not been investigated due to constraints on DNA availability, genomic deletion is the dominant cause of PTEN decrease in human melanoma (Madhunapantula and Robertson, 2009). Representative cases in which PTEN locus is intact and PTEN protein expression is retained (a) or at least a portion of PTEN locus is completely deleted and PTEN protein expression is lost (b) are shown in Figure S3.

BRAF mutation was detected in 20 out of the 28 lines (71.4%) (Figure 2a). SK-Mel-85 cell line is the only one showing mutation at the G468 residue in exon 11, while in all the remaining lines the mutation involves the V599 residue in exon 15. The rate of BRAF mutation (V599) in melanoma tissues was 30.2% (13 out of 43), 21.7% in the primary samples (5 out of 23) and 40% in the metastatic samples (8 out of 20) (Figure 2b). NRAS mutation (Q61 residue in exon 3) was detected in 4 out of 28 cell lines (14.3%) and was mutually exclusive with BRAF mutation, as reported elsewhere (Tsao et al., 2004). No mutations in NRAS were found in the melanoma tissues. The low rate of BRAF and NRAS mutation in metastatic samples is consistent with the prevalence of lymph node as metastatic site, as previously reported by our group (Gorden et al., 2003). As reported elsewhere (Gast et al., 2010), we found that partial or complete PTEN deletion frequently occurs in concomitance with BRAF mutation (in 10 out of 13 cases (76.9%) in melanoma cell lines and in 9 out of 23 cases (39.1%) in melanoma tissues, Figure 2). The coexistence of partial or complete PTEN deletion and NRAS mutation occurred at much lower frequency (Tsao et al., 2000). Among the 13 cell lines where PTEN is deleted, only WM1361A and Sk-Mel-103 (2 out of 13, 15.4%) showed both PTEN deletion and NRAS mutation.
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SUPPLEMENTAL TEXT S3

Materials and Methods

Melanoma cell lines. Normal human melanocytes (NHM) were obtained from infant foreskin and grown in supplemented melanocyte growth medium (PromoCell, Germany). Primary human melanoma cell lines (WM35, WM1552c, WM1575, WM3211, WM98.1, WM115, WM278, WM793B, WM853.2, WM902B, WM983A, WM1366, WM1361A, WM3248) were purchased from the Wistar Institute and were cultured in Mel 2% medium (Fang et al., 2005). Sk-Mel-19, Sk-Mel-29, Sk-Mel-85, Sk-Mel-94, Sk-Mel-100, Sk-Mel-103, Sk-Mel-147, Sk-Mel-173, Sk-Mel-187, Sk-Mel-192 and Sk-Mel-197 metastatic human melanoma cell lines were a kind gift from A.N. Houghton (MSKCC, New York). 501mel metastatic human melanoma cell line was obtained from Yale University. A375 and 451Lu metastatic human melanoma cell line were purchased from the American Type Culture Collection and the Wistar Institute, respectively. All the metastatic melanoma cell lines were cultured in DMEM +10%FBS.

Melanoma tissues. Patients used in this study were receiving treatment at New York University (NYU) Langone Medical Center. They were prospectively enrolled into the Interdisciplinary Melanoma Cooperative Group (IMCG) database (Wich et al., 2009) since August 2002. All patients provided written consent at enrollment and the Internal Review Board of NYU approved the study. Information about the characteristics of the primary and metastatic tissues were collected and are reported in Table S1. The study was conducted according to Declaration of Helsinki Principles.

Primer design for genomic qPCR. All gene-specific primers were designed using Primer3 and the PCR product size ranged between 80 and 150bp (Table S2). The ~4kbp PTENP1 locus was amplified using primers located in the downstream half of
its 3'UTR, which shows low sequence similarity with the corresponding PTEN region (R2 region in Figure 1a). The 5', middle and 3' regions of the ~109kbp PTEN locus were amplified using three sets of primers: the first is located at the beginning of intron 1, the second at the beginning of intron 3 and the third in the most downstream region of the 3'UTR in exon 9 (R3 region in Figure 1a). The fragments amplified by these 3 primer sets are all absent in PTENP1, the first two because PTENP1 is an intron-less, processed pseudogene (D'Errico et al., 2004) and the last because the 3'UTR of PTENP1 is shorter than the one of PTEN (Figure 1a). Using this primer design, we have therefore been able to monitor the whole PTEN locus and at the same time to avoid cross-amplifications from the PTENP1 locus.

For CDKN2A detection, we used a set of primers located on exon 2, which contains coding information required for the translation of both the protein products expressed from this locus: the cell cycle regulator p16 and the MDM2 inhibitor p14/ARF (Freedberg et al., 2008; Sharpless and Chin, 2003).

For CDKN2B and MTAP detection, the primers were positioned in exon 1 and 5, respectively.

**Genomic qPCR.** Genomic DNA was extracted from pellets of melanoma cell lines and macrodissected melanoma tissue slides. The QIAamp DNA mini kit (Qiagen, Germany) and the FormaPure kit (Agencourt, Beverly) were used, respectively. We estimate that all tissue samples consisted of ≥90% melanoma cells. PCR reactions were performed using MyiQ Real time PCR instrument (BioRad, Hercules) in 96-well plates, with reaction mixture (25 µL) containing 20 ng of genomic DNA the specific primers and 1x BIO-RAD IQTM SYBR Green Supermix. To determine the genetic load, the ΔΔCT method was used (Pfaffl, 2001) assuming 100% efficiency in the amplification and using normal human genomic DNA (Promega, Madison) and normal human melanocytes as standard
for the melanoma cell lines. For the human melanoma samples the average of the results obtained using genomic DNA extracted by 2 different congenital nevi was instead used. Two housekeeping genes, \textit{UBE2E1} and \textit{GNS}, were used as reference as previously described (Lazar et al., 2009). The deletions of the loci under study were considered partial if they ranged between 80% and 10% of the references, complete if they were below 10%. qPCR data were analyzed with unpaired t test (GraphPadPrism, GraphPad Software Inc., San Diego). Values of p < 0.05 were considered statistically significant (*p < 0.05; **p < 0.01; ***p < 0.001). The mean ± s.d. of three independent experiments is reported.

**Mutation analysis.** BRAF exon 11 (encoding for G465 and G468 residues), BRAF exon 15 (encoding for V599 residue), NRAS exon 2 (encoding for G12 and G13 residues) and NRAS exon 3 (encoding for Q61 and Y64 residues) were amplified from the genomic DNA extracted from melanoma cell lines and tissues. Phusion Flash High-Fidelity PCR Master Mix (Finnzyme, Waltham) and the primers listed in Table S3 were used. PCR products were run on 1.5% agarose gel and purified using the QIAquick Gel Extraction kit (Qiagen, Germany) and then subjected to sequencing (GENEWIZ DNA sequencing service). The resulting traces were analyzed using 4Peaks software. Each PCR fragment was sequenced using both the forward and the reverse primers. For some of the cell lines, the sequencing results reported in ref. (Gorden et al., 2003; Mills et al., 2009; Primot et al., 2010) and at http://www.wistar.org/ were used.

**Western blot.** Cells were collected and lysed (50mM Tris pH8.0, 1mM EDTA, 1mM MgCl$_2$, 150mM NaCl, 1% NP-40, 1 mM $\beta$-glycerophosphate, 1 mM Na$_2$VO$_4$, 1 mM NaF, protease inhibitors). Proteins (30 $\mu$g/lane) were separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Immunoblotting of the membranes was
performed using the following primary antibodies: anti-PTEN (#9559, Cell Signaling, Danvers, 1:1000), anti-Ran (#sc-1156, Santa Cruz Biotechnology, Santa Cruz, 1:200). Signals were revealed after incubation with recommended secondary antibody coupled to peroxidase by using enhanced chemiluminescence.

**Immunohistochemistry (IHC).** Primary and metastatic melanoma tissues were deparaffinized and rehydrated, subjected to Heat Induced Epitope Retrieval (HIER), using a 2.37 l microwave safe pressure cooker (Nordik Ware, Minneapolis) containing 10 mM Sodium citrate, 0.05% Tween 20, pH 6.0 solution. The pressure cooker was placed in a 1200 w microwave oven at full power for 20 min. The slides were then placed in aqueous 0.3% H$_2$O$_2$ solution for 30 min to quench the endogenous peroxidase. The primary antibody (anti-PTEN rabbit polyclonal antibody, #18-0256, Invitrogen, Carlsbad, 1:50) was incubated for 1h at room temperature followed by an overnight incubation at 4ºC. The secondary antibody (biotinylated goat anti-rabbit, Vector Labs, Burlingame) and the VECTASTAIN Elite ABC Kit (Vector Labs, Burlingame) were used accordingly with manufacturer’s instructions. Positive and negative control slides were included in each experiment. Blinded to patients’ clinical data, an attending pathologist (F.D.) scored the expression of PTEN in each tissue. Expression of PTEN was scored according to proportion of cells with positive cytoplasmic staining. A cutoff of 50% of cells showing PTEN immunoreactivity was established based on data showing that PTEN is haploinsufficient in tumor suppression and that its dose is a key determinant in cancer progression (Berger and Pandolfi, 2011).

**Statistical analysis.** Associations between PTEN deletion status and CDKN2A deletion status or PTEN protein expression (IHC) were explored by Fisher’s exact test. p-values
are one-sided with statistical significance evaluated at the 0.05 alpha level. All analyses were performed in SPSS Version 18.0 (SPSS Inc., Chicago).

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|       | CDKN2A  |
|-------|---------|
|       | no del  | del   |
| **PTEN** |         |       |
| no del | 16 (80%) | 4 (20%) |
| del    | 11 (47.8%) | 12 (52.2%) |

**a**

\[ p = 0.03^* \]

|       | PTEN positive cells (IHC) |
|-------|---------------------------|
|       | ≤50% | >50% |
| **PTEN** |         |       |
| no del | 16 (80%) | 4 (20%) |
| del    | 23 (100%) | 0 (0%) |

**b**

\[ p = 0.04^* \]

Supplementary Figure 1. a. Correlation between PTEN deletion and CDKN2A deletion in the 43 melanoma tissues. b. Correlation between PTEN deletion and PTEN protein level in the 43 melanoma tissues. In both a and b, tissues were considered as PTEN deleted if showing partial or complete deletion in any of the 3 regions tested by genomic qPCR (intron 1, intron 3, exon 9). *Fisher exact test (1 tailed).
**Supplementary Figure 2. PTEN loss in melanoma cell lines.**

**a.** Genomic qPCR analysis of PTEN intron 1 (upper), intron 3 (middle) and exon 9 (lower) reveals complete deletion in the primary cell lines WM98.1 and WM853.2, as well as in the metastatic cell lines Sk-Mel-85 and Sk-Mel-94 (highlighted). On the contrary, the deletion is only partial in the primary cell line WM35 and the metastatic cell line Sk-Mel-103. The “wild type” WM902B and 451Lu cell lines are reported as reference. The mean ± s.d. of 3 independent experiments is reported. The statistically significant deletions are indicated. *p < 0.05; **p < 0.01; ***p < 0.001.

**b.** Western blot analysis of the same cell lines shown in **a** confirms the absence of PTEN protein only in WM98.1, WM853.2, Sk-Mel-85 and Sk-Mel-94, while WM35 and Sk-Mel-103 show residual protein expression.
Supplementary Figure 3. PTEN genomic status and protein level in representative primary and metastatic melanoma tissues. a. The primary melanoma tissues P22 and P23 and the metastatic melanoma tissue M12 retain an intact PTEN locus (genomic qPCR, upper). The presence of PTEN protein in P23 is shown in the lower panels (immunohistochemistry, IHC). b. The primary melanoma tissues P18 and P20 and the metastatic melanoma tissue M14 show complete deletion of PTEN locus (genomic qPCR, upper). The loss of PTEN protein in P18 is shown in the lower panels (IHC). i1: intron 1; i3: intron 3; E9: exon 9. The mean ± s.d. of 3 independent experiments is reported. *p < 0.05; ***p < 0.001.
### Supplementary Table 1. Characteristics of primary (a) and metastatic (b) patients.

|                     | N=23        | N=20        |
|---------------------|-------------|-------------|
| **Gender N (%)**    |             |             |
| Male                | 15 (65.2)   | 12 (60)     |
| Female              | 8 (34.8)    | 8 (40)      |
| **Age at Diagnosis, years** | 70 (29-92) | 63.8 (26-82)|
| median (range)      |             |             |
| **Stage N (%)**     |             |             |
| Stage I             | 0 (0)       | 15 (75)     |
| Stage II            | 14 (60.9)   | 5 (25)      |
| Stage III           | 9 (39.1)    |             |
| **Thickness, mm**   | 4.5 (1.4-24)|             |
| median (range)      |             |             |
| **Ulceration N (%)**|             |             |
| Present             | 18 (78.3)   | 5 (25)      |
| Absent              | 5 (21.7)    | 13 (65)     |
| **Mitotic index N (%)** |         |             |
| Present             | 21 (91.4)   | 2 (10)      |
| Absent              | 1 (4.3)     |             |
| Unknown             | 1 (4.3)     |             |
### Supplementary Table 2. Primers used for qPCR on genomic DNA extracted from melanoma cell lines and tissues.

| Locus       | Forward primer            | Reverse primer            |
|-------------|---------------------------|---------------------------|
| **CDKN2A**  | TTCCAGCTTTCTAGTGACCAT     | CCCCAATGTCTATGTCTCTAA     |
| **CDKN2B**  | TACCCAGCAAAAAATCCTAAA     | GCTTTCCCTTTTCTCCTTTTC     |
| **MTAP**    | TCTTGTGCCAGAGGAGTGTG      | CCCAAGCCCACATGATTTAT      |
| **PTEN intron 1** | GTTAGTGGTCATCGAAAAGC          | TGCAATACCTTTCCCATTCC     |
| **PTEN intron 3** | TCAAGAAGTCCAAGAGCATT            | GCTCAATATGGGGCTAGATG       |
| **PTEN exon 9** | TGGGGGCTTTAACTGTAGTA             | GGATGAGGCAATTATCCTGTA      |
| **PTENP1**  | TCAGAACATGGCATAACCCAA      | TGATGACGTCGATTATTTTCA     |
| **GNS (ref)** | TCCAACTTTTGAGCCCTTCTT      | CGTTCCATGGATGGTAAGT        |
| **UBE2E1 (ref)** | GGTGGGAAGTATGGCACCACTCA             | GTGAAACCCCAATTATGTAGCGTAT |

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Supplementary Table 3. Primers used for the amplification and sequencing of BRAF exon 11 and 15 and NRAS exon 2 and 3.

| Exon                  | Forward primer          | Reverse primer          | PCR product (bp) |
|-----------------------|-------------------------|-------------------------|------------------|
| BRAF exon 11 (cell lines) | TCCCTCTCACCCATAGGTAA    | CGAACAGTGAATTTCTTTGAT   | 313              |
| BRAF exon 11 (tissues) | GGTAGACGGGACTCAGT      | TACTTACCATGCCACTTCC     | 116              |
| BRAF exon 15 (cell lines) | TCATAATGCTTGCTGTAGGA    | GGCCAAAAATTTAATCAGTGA  | 224              |
| BRAF exon 15 (tissues) | GACCTCAGGATAATAGGGT     | GGCCAAAAATTTAATCAGTGA  | 144              |
| NRAS exon 2 (cell lines) | GAACAAATGGAGGTCACA      | TGGGAAAGATGATCCGACA     | 301              |
| NRAS exon 2 (tissues)  | GACCTCAGGATAATAGGGT     | GGCCAAAAATTTAATCAGTGA  | 144              |
| NRAS exon 3 (cell lines and tissues) | CATACTGGATACAGCTGGAC    | TGACTTGCTATTATGGATGG    | 106              |

1 Primer sequences taken from (Davies et al., 2002)
2 Primer sequences taken from (Gorden et al., 2003)

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