Tumor-derived inducible heat-shock protein 70 (HSP70) is an essential component of anti-tumor immunity

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The anti-apoptotic function and tumor-associated expression of heat-shock protein 70 (HSP70) is consistent with HSP70 functioning as a survival factor to promote tumorigenesis. However, its immunomodulatory activities to induce anti-tumor immunity predict the suppression of tumor growth. Using the Hsp70.1/3−/− (Hsp70−/−) mouse model, we observed that tumor-derived HSP70 was neither required for cellular transformation nor for in vivo tumor growth. Hsp70−/− murine embryonic fibroblasts (MEFs) were transformed by E1A/Ras and generated tumors in immunodeficient hosts as efficiently as wild-type (WT) transfectants. Comparison of Bcr-Abl-mediated transformation of WT and Hsp70−/− bone marrow and progression of B-cell leukemogenesis in vivo revealed no differences in disease onset or survival rates, and Eμ-Myc-driven lymphoma in Hsp70−/− mice was phenotypically indistinguishable from that in WT Eμ-Myc mice. However, Hsp70−/− E1A/Ras MEFs generated significantly larger tumors than their WT counterparts in C57BL/6 J immune-competent hosts. Concurrent with this was a reduction in intra-tumoral infiltration of innate and adaptive immune cells, including macrophages and CD8⁺ T cells. Evaluation of several potential mechanisms revealed an HSP70-chemokine-like activity to promote cellular migration. These observations support a role for tumor-derived HSP70 in facilitating anti-tumor immunity to limit tumor growth and highlight the potential consequences of anti-HSP70 therapy as an efficacious anti-cancer strategy.

INTRODUCTION

Heat-shock proteins (HSPs), the functional components of the inducible heat-shock response, are implicated in the regulation of tumorigenesis by virtue of their ability to promote tumor cell survival.¹ However, the only in vivo genetic evidence of a pro-tumorigenic role for the stress response is the abrogation of tumor formation in mice deficient for heat shock factor-1 (HSF-1), the transcription factor essential for the expression of multiple HSPs.²,³ Nevertheless, evidence suggests that ‘addiction’ to components of the stress response, including HSP70, may sustain tumor survival and drive tumor growth.¹,⁴

HSP70 also regulates immune function, including antigen cross presentation,⁵,⁶ dendritic cell maturation⁷,⁸ and natural killer (NK) cell⁹,¹⁰ and myeloid-derived suppressor cell¹¹ activities. Extra-cellular HSP70 regulates these diverse immunoregulatory activities by acting as a cytokine to stimulate the release of pro-inflammatory factors from immune cells or from tumor cells.¹³ HSP70 is released from tumor cells via passive release from dying cells and active trafficking via the endolysosomal pathway¹⁴ or release within lipid-bound exosomes.¹¹,¹⁵

Discrimination between a need for tolerance and the demand for immunity represents a fundamental principal of maintaining immunological homeostasis. Tolerance prevents autoimmunity but because of the extensive overlap of self-peptides with tumor-associated antigens, also suppresses anti-tumor immunity. The immune system can distinguish ‘normal’ from ‘abnormal’ self to overcome tolerance and instead invoke immunity via mechanisms, such as the release of immunogenic ‘danger signals’,¹⁶ that include HSPs.¹⁷,¹⁸ Although HSPs may be critical determinants of a need for tolerance or circumstances requiring an immune response,¹⁹,²⁰ it remains controversial whether this is mediated by promoting immunity or by suppressing immune responses to maintain tolerance.¹⁹,²¹

To date, no studies have utilized the Hsp70.1/3−/− murine model to address whether HSP70, like HSF-1,²,³ is a critical pro-survival signal for tumor cells in vivo or to evaluate the consequences of HSP70-mediated immune regulation in the context of anti-tumor immunity and tumor growth in situ. Clearly, HSP70 can contribute to multiple aspects of immune regulation, but it remains unclear if this manifests in the suppression of tumor growth by activation of anti-tumor immunity,²²,²³ or immunosuppression to exacerbate tumorigenesis.¹⁹

We utilized the Hsp70.1/3−/− murine model, in which both alleles of inducible HSP70 are deleted,²⁴ to ask whether HSP70 is essential for oncogene-induced transformation; whether HSP70 has a non-redundant role in tumor growth in vivo; and whether the immunoregulatory activity of HSP70 inhibits or promotes tumor growth in vivo? We present data here that challenge an essential pro-tumorigenic role for tumor-derived HSP70 but instead support a model in which it negatively regulates tumor growth in vivo by engaging T-cell dependent immunity. For the first time, using the Hsp70.1/3−/− murine model, we demonstrate that HSP70 is a non-essential pro-tumorigenic factor but instead functions as a danger signal to facilitate anti-tumor immunity and suppress tumor growth in vivo.

RESULTS

HSP70 is neither required for oncogene-induced transformation in vitro nor tumor growth in vivo

Wild-type (WT) or Hsp70−/− murine embryonic fibroblasts (MEFs) transduced with E1A and Ras, but not empty vector or E1A or
Ras alone, generated drug-resistant colonies of approximately equivalent number (Figure 1a). However, those colonies lacking Hsp70 appeared larger in size, although no difference in the in vitro growth rates of WT and Hsp70−/− MEFs was observed (not shown). Consistent with observations using immortalized HSF-1−/− primary HSF-1−/− MEFs did not transform (Figure 1a). Quantitative PCR of Hsp70 expression confirmed the genotype of emergent clones (Figure 1b). WT and Hsp70−/− E1A/Ras transformants generated tumors in immunodeficient mice with no significant difference in tumor size (Figures 1c and d). Similar data were obtained using independently generated WT and Hsp70−/− E1A/Ras transformants. All tumors were classified as sarcomas (Supplementary Figures S1A and B), and neither the frequency of karyomegaly nor mitoses revealed any significant difference between WT and Hsp70−/− tumors (Supplementary Figure S1C).

Selection for Hsp70 expression in WT tumors

WT tumors from CD1-Foxn1nu mice had elevated Hsp70 gene expression, compared with the MEFs used for inoculation (Figure 1e). Hsp70−/− tumors were not completely devoid of Hsp70 expression, presumably due to host-derived contamination such as vasculature (Figure 1e), and Hsp90AA1 gene expression in WT and Hsp70−/− tumors was comparable and largely unchanged from that in the MEFs used for inoculation (Figure 1f).

Figure 1. Hsp70 is neither required for in vitro transformation nor tumor growth in vivo. WT, HSF-1−/− or Hsp70−/− primary MEFs were transduced with empty vector control, E1A or Ras alone or both E1A and Ras retroviral vectors as indicated, and after re-plating (4 × 10⁴–2×10⁵/well) and antibiotic selection for approximately 14 days, colonies were visualized by methylene blue staining (a). Quantitative PCR for Hsp70 (normalized to L32 expression) of emergent colonies (b). WT or Hsp70−/− E1A/Ras transformants were injected subcutaneously into each of the flanks of CD1-Foxn1nu mice, and tumor growth monitored by ultrasound. Volumetric tumor measurements (mm³) were derived from 3-D reconstructions of the ultrasound data sets. Data are shown as the average (n = 5–10 mice/group) ± s.d. and is representative of six independent experiments (c). Representative images of one tumor (marked with asterisk (*) in panel (c)) in which the left panels show 2-D slices of the tumor and the right panels show the 3-D volumetric reconstruction (d). Relative expression of Hsp70 (e) and Hsp90AA1 (f) normalized to L32. Asterisks denote the expression levels of Hsp70 and Hsp90AA1 in the MEFs used for inoculation.
Neither Bcr-Abl nor Eμ-Myc requires HSP70 to induce leukemia
We also examined the requirement for HSP70 in (i) Bcr-Abl-induced leukemia25 and (ii) Eμ-Myc-induced B-cell lymphoma.26 WT and Hsp70−/− bone marrow (BM) was transduced with a retroviral vector expressing Bcr-Abl-GFP before culture in vitro or transfer into lethally irradiated C57BL/6 J WT hosts. WT and Hsp70−/− BM cultures proliferated at similar rates before (Figure 2a) and after (Figure 2b) removal from stromal support. Equivalent green fluorescent protein (GFP) expression was detected in the WT and Hsp70−/− BM transformants (Figure 2c), and evaluation of Hsp70 gene expression confirmed genotype specificity (Figure 2d). Similar disease onset was observed in animals receiving WT or Hsp70−/− BM, with no significant difference in overall survival rates (Figure 2e). No genotype-specific differences were observed in spleen weight (Figure 2f) or percentage of GFP+ cells in the BM, spleen and blood (g). Lymphoma-free survival using a Log Rank Mantel Cox test (Prism software) and median survival (in days) (i). Relative frequencies (expressed as a percentage) of IgM− pre-B-cell lymphomas and IgM+ mature B-cell lymphomas as determined by the percentage of IgM staining of the B220+ population in the spleen (< 50% IgM+ was assigned a pre-B phenotype) (k).

Figure 2. Neither Bcr-Abl nor Eμ-Myc requires HSP70 to induce lymphoma. BM from each of two WT and two Hsp70−/− mice was transduced with a retroviral vector expressing Bcr-Abl-GFP. Cell growth was monitored before (a) and after cells were removed from the stromal layer (b). After approximately 2 weeks, GFP expression was assessed via flow cytometry (c) and Hsp70 gene expression via quantitative PCR (d). Survival of the two cohorts receiving Bcr-Abl-GFP transduced WT or Hsp70−/− BM (e), spleen weight (f) and percentage of GFP+ cells in the BM, spleen and blood (g). Lymphoma-free survival of WT, Hsp70−/− and Hsp70−/− Eμ-Myc mice (h) and average spleen weight (j). Statistical evaluation of lymphoma-free survival using a Log Rank Mantel Cox test (Prism software) and median survival (in days) (i). Relative frequencies (expressed as a percentage) of IgM− pre-B-cell lymphomas and IgM+ mature B-cell lymphomas as determined by the percentage of IgM staining of the B220+ population in the spleen (< 50% IgM+ was assigned a pre-B phenotype) (k).

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Tumor-derived HSP70 only retards tumor growth in an immune-competent host

To specifically address the role of intra-tumoral HSP70, we further utilized the MEF-derived sarcoma model. WT E1A/Ras cells introduced into immune-competent hosts formed small tumors that subsequently regressed, whereas Hsp70−/− transformants generated significantly larger tumors (Figures 3a and b). A total of 32 WT C57BL/6 J mice (in three independent experiments) and 55 CD1-Foxn1nu mice (in six independent experiments) were used to assess in vivo tumor growth. One representative C57BL/6 J experiment is shown in (a), and for comparison, two independent CD1-Foxn1nu experiments are shown in panel (c) and Figure 1c. HSP70 expression in tumors from C57BL/6 J mice was determined via immunostaining (d). The scale bars correspond to 250 or 50 microns in the ×10 and ×40 images, respectively. RNA from the tumors was used to determine the expression of Hsp70 (e), Hsp90AA1 (f) and Hsc70 (g) via quantitative PCR. Relative gene expression is shown, and all data were normalized to the control gene L32. Left (L) and right (R) flank tumors are numbered sequentially, and asterisks denote the expression levels in the transformants used for inoculation.

HSP70 expression in WT tumors that was largely absent from Hsp70−/− tumors (Figure 3d and Supplementary Figures S1F and G). Hsp90AA1 expression was increased in Hsp70−/− but not WT tumors (Figure 3f), and constitutive Hsc70 remained at or below control levels in WT and Hsp70−/− tumors (Figure 3g).

Hsp70−/− tumors display a marked reduction in the infiltration of immune cells

WT tumors from C57BL/6 J hosts displayed macrophage infiltration throughout the tumor mass while those lacking Hsp70 showed a reduction in MAC-2 staining that was largely restricted to the tumor periphery (Figure 4a and Supplementary Figures S2C).
and D). Although Hsp70−/− tumors from CD1-Foxn1nu hosts also showed a reduction in macrophage number compared with WT tumors, they were distributed throughout the tumor mass (Figure 4b and Supplementary Figures 2A and B).

Differential growth of WT and Hsp70−/− tumors in C57BL/6 J hosts compared with equivalent growth in CD1-Foxn1nu mice suggests that HSP70 may suppress tumor growth in a T-cell-dependent manner. Indeed, WT tumors displayed extensive intra-tumoral lymphocytic infiltration, whereas Hsp70−/− tumors were characterized by a reduced number of CD3+ cells, largely restricted to the tumor periphery (Figure 4c and Supplementary Figure S3). Extensive intra-tumoral CD3+/CD4+ (Figure 5a) and CD8+/GrB+ (Figure 5b) co-staining was observed in WT tumors that was reduced and largely undetectable in Hsp70−/− tumors. NKT cells were detected in WT and, to a lesser extent, in Hsp70−/− tumors (Figure 5c) and while WT tumors had extensive granzyme B and perforin expression, Hsp70−/− tumors exhibited a reduction in the expression of both (Figures 5d and e and Supplementary Figures 4D and G).

Knockdown of Hsp70 abrogates intra-tumoral immune cell infiltration and promotes tumor growth

WT MEFs stably expressing Hsp70 or control shRNAs vectors co-expressing red fluorescent protein (RFP) were evaluated for their ability to generate tumors in vivo. Hsp70 shRNAs reduced HSP70 protein levels while RFP expression was equivalent in both control and Hsp70 shRNA expressing cells (Figure 6a and Supplementary Figures S5A and B). Tumors expressing Hsp70 or control shRNAs were equal in size in immunodeficient mice (Figure 6b), but in C57BL/6 J hosts, cells expressing Hsp70 shRNA generated larger tumors compared with those expressing control shRNAs (Figure 6d). Furthermore, Hsp70 shRNA tumors from immune-competent mice displayed a selective retention of RFP and reduced HSP70 proteins (Figure 6e and Supplementary Figures S5I and L). In contrast, RFP protein levels were variable in tumors isolated from CD1-Foxn1nu hosts, with no preferential expression in the Hsp70−/− tumors (Figure 6c and Supplementary Figures S5C and H). Hsp70 shRNAs expressing tumors displayed a marked reduction in macrophage and T-cell infiltration as...
compared with control shRNA tumors (Figures 6f and h and Supplementary Figures 5M and 5N, respectively). In contrast, macrophage distribution was indistinguishable between WT and Hsp70−/− tumors harvested from CD1-Foxn1nu hosts (Figure 6g and Supplementary Figure S5O). These data confirm that tumor growth in vivo is inhibited by Hsp70-dependent immunoregulation that, paradoxically, is opposed by an immune-dependent selection against HSP70 expression (Figure 6e).

Tumor-derived HSP70 functions in a chemokine-like manner to facilitate intra-tumoral infiltration of immune cells HSP70 can augment antigen cross presentation,18 consistent with suppression of T-cell activation and exacerbation of tumor growth observed in our Hsp70−/− model. However, BM-derived dendritic cells, were equally capable of cross-presenting irradiated WT and Hsp70−/− MEFs preloaded with Class I MHC SIINFEKL peptide as determined via transgenic OT-I CD8+ T-cell proliferation (Supplementary Figure S6A). Evaluation of the number and activity of NK and NKT cells found neither to be lacking in WT hosts harboring Hsp70−/− tumors (Supplementary Figure S6B), and characterization of phenotypic and functional features shared by tumor associated macrophages and myeloid-derived suppressor cells, both of which can augment tumor growth, revealed no difference in frequency or distribution as compared with MAC-2 staining (Supplementary Figures S6C and D). Therefore, three modes of immune activity that can be regulated by HSP70 could not account for the exacerbation of Hsp70−/− tumor growth that we observed.

WT or Hsp70−/− E1A/Ras transformants were each introduced into cohorts of WT and Hsp70−/− C57BL/6J hosts, and tumor growth was monitored. WT MEFs generated significantly smaller tumors compared with their Hsp70−/− counterparts regardless of the host genotype, and exacerbated growth of Hsp70−/− tumors in WT mice was also observed in Hsp70−/− hosts (Figure 7a). Furthermore, the reduction in WT tumors in either WT or Hsp70−/−
hosts correlated with a pronounced infiltration of immune cells while the larger $Hsp70^{-/-}$ tumors were instead characterized by a largely peripheral and minimal intra-tumoral distribution of macrophages and T cells in both WT and $Hsp70^{-/-}$ hosts (Figures 7b and c, respectively). We conclude that tumor-derived HSP70 is impacting tumor growth independent of intrinsic tumor cell survival but by regulating immune cell infiltration in a localized manner.

Splenocytes from WT C57BL/6J mice inoculated 5–7 days before with WT or $Hsp70^{-/-}$ MEFs were co-cultured with WT or $Hsp70^{-/-}$ E1A/Ras MEFs, and after 4 h, the number of splenocytes in the lower chambers was enumerated. WT splenocytes primed in a host harboring a WT tumor migrated at a frequency proportional to the number of WT MEFs plated in the lower chamber (Figure 7d). In comparison, the migration of WT splenocytes primed in vivo by $Hsp70^{-/-}$ MEFs occurred at significantly reduced numbers that inversely correlated with the number of $Hsp70^{-/-}$ MEFs (Figure 7d). Strikingly, WT splenocytes primed by WT tumors failed to migrate towards $Hsp70^{-/-}$ MEFs, and in the reciprocal scenario, WT splenocytes primed by $Hsp70^{-/-}$ tumors were not stimulated to migrate by WT MEFs (Figure 7d). Importantly, naive splenocytes with no previous in vivo priming migrated towards WT and $Hsp70^{-/-}$ MEFs in equivalent numbers (Figure 7e). Similar findings were observed using conditioned media collected from overnight cultures of WT or $Hsp70^{-/-}$ MEFs (not shown). These observations are consistent with the idea that tumor-derived HSP70 can function as a soluble chemokine within the localized tumor microenvironment to facilitate intra-tumoral immune cell infiltration.

**DISCUSSION**

We have found that, remarkably, both alleles of inducible HSP70 were dispensable for oncogenesis and tumor growth in the three...
models of transformation. Further, although the absence of HSP70 was irrelevant for growth of transformed MEF in immunocompromised hosts, it resulted in defective immunity and enhanced tumor growth in immunocompetent animals. These studies are the first to utilize a genetically defined model and allowed us to isolate the requirement for HSP70 as an endogenous factor to sustain tumor cell survival and its extra-tumoral role to modulate the host immune response. Clearly HSP70 can sustain tumor survival and regulate immune function, but we suggest that in those cases where HSP70 is dispensable for oncogenesis it may...
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instead contribute to anti-tumor immunity. Understanding the mechanisms through which HSP70 can regulate tumorigenesis in situ is critical if efforts to exploit the stress pathway as an anti-cancer strategy are to succeed.27

HSP70 is neither required for oncogene-driven transformation nor for tumor growth in vivo in the three independent in vivo models (Figures 1 and 2) and opposes the idea that tumor-derived HSP70 is absolutely necessary for tumor cell survival. It is possible, however, that WT tumors acquire a dependency for HSP70 expression, whereby its disruption could provoke tumor cell death.28 However, E1A/Ras transfectants stably expressing Hsp70 shRNAs formed tumors in vivo (Figures 6b and d), implying that cells transformed in the presence of HSP70 not only survive but also remain with their equivalent growth in immunodeficient hosts (Figures 1c and 3c). Hsp70−/− tumors were characterized by reduced intra-tumoral immune infiltrate (Figures 4 and 5), suggesting that tumor-derived HSP70 can influence the recruitment, retention or activation of anti-tumor immune cells.

Although HSP70 can promote the activity of NK cells,31 we observed no decrease in Hsp70−/− tumors (Figure 5). In addition, both NK (CD3− NK1.1+) and NK (CD3− NK1.1+) cell numbers and activity were increased in mice harboring Hsp70−/− tumors (Supplementary Figure S6B). Although the reason for this is unclear, it cannot explain the increased growth of Hsp70−/− tumors but is consistent with HSP70-mediated immune suppression under some circumstances.21,22 Quantification of T cells in tumor-bearing WT mice did not reveal preferential accumulation of any sub-type based on the HSP70 status of the tumor. Interestingly, however, whereas the CD3− population appeared largely confined to the periphery of Hsp70−/− tumors, Foxp3+ cells were distributed throughout (Supplementary Figures S4A and B). HSP70 can enhance the immunosuppressive activity of FOXP3+ regulatory T cells,29,30 and although we cannot exclude this possibility, it is inconsistent with enhanced Hsp70−/− tumor growth. Analysis of tumor-associated macrophages and myeloid-derived suppressor cells16 also failed to reveal any skewing of growth. Analysis of tumor-associated macrophages and myeloid-sustained HSP70 as a determinant of the immunoediting process.35 deletion of tumor cells expressing the highest HSP70 levels nomimates HSP70 as a determinant of the immunoediting process. Therefore, the selection for HSP70 sustains tumor immunogenicity, vulnerability to lymphocyte attack and tumor cell deletion. Consistent with this, HSP70 tumor content decreases as tumors advance, and high HSP70 levels provide a prognostic indicator of survival.35,37

HSP70 facilitates antigen cross presentation to engage CD8+-dependent cytolytic activity. However, BM-derived dendritic cells were equally capable of cross-presenting antigens from WT and Hsp70−/− E1A/Ras transfectants (Supplementary Figure S6A), and reciprocal implantation of WT or Hsp70−/− tumor cells into Hsp70−/− or WT hosts, respectively, produced tumors and immune infiltration indistinguishable from their growth in hosts of the same genotype (Figures 7a and c). Collectively, these data are inconsistent with HSP70 within the tumor itself or in peripheral antigen-presenting cells facilitating antigen cross presentation.

Extracellular HSP70 can regulate immune cells11,14,31 to suppress tumor growth.29 Splenocytes pre-primed in vivo by WT tumors migrated towards WT transfectants, whereas those pre-primed by Hsp70−/− tumors were unable to migrate towards Hsp70−/− tumor cells (Figure 7d). Similar observations were made using conditioned media collected from WT or Hsp70−/− MEFs, suggesting that a soluble factor was mediating this activity. However, the dependence of splenocyte migration upon pre-priming (Figure 7e) suggests that HSP70 also imparts migratory activity to immune cells within the tumor microenvironment. Inhibition of the migratory capacity of splenocytes from mice harboring WT tumors by co-culture with Hsp70−/− splenocytes (Figure 7d) suggests that Hsp70−/− tumor cells are deficient in a stimulatory factor that functions either directly or indirectly.

Using the Hsp70+1.3−/− model, we demonstrate a non-critical tumor intrinsic role for HSP70 in tumor growth in vivo. Instead, HSP70 represents a critical component for tumor recognition by the adaptive immune system and promotes host immunity in situ to promote tumor destruction.

MATERIALS AND METHODS

Additional details are included in Supplementary Information.

Animals

All mice were cared for in accordance with the NIH guidelines and adhering to procedures approved by St Jude Children’s Research Hospital Animal Care and Use Committee (IACUC). Genotyping was conducted according to the published methods37 and online at jaxmice.jax.org.

Preparation, in vitro transformation and maintenance of MEFs

Timed heterozygous breeding between appropriate genotypes was used to generate embryos WT or null for HSF-1 or Hsp70. Retroviral particles were produced using the ecotropic Phoenix packaging cell line with the following plasmids: pWZL-hygro; pBABE-puro; pWZL-hygro 125 E1A; or pBABE-puro K-Ras V12 (purchased from Addgene, Cambridge, MA, USA). Primed WT or Hsp70−/− MEFs were infected with virus for 6 h before medium replacement, antibiotic selection and colony outgrowth visualized by methylene blue staining. In some experiments, transformed MEFs were maintained in culture for experimentation—the genotype was confirmed by quantitative PCR and cell lines were maintained in vitro for a maximum of 10 passages before rescue of an earlier passage from liquid nitrogen storage. All cell lines generated were tested approximately every 3 months for mycoplasma contamination using MycoSEQ Mycoplasma Detection Assay (Invitrogen, Carlsbad, CA, USA) and discarded if found to be positive.

MEF tumor implantation

WT or Hsp70−/− E1A/Ras MEF transformants were introduced subcutaneously into each of the flanks of CD1-Foxn1−/−, C57BL/6 J or Hsp70−/− mice, and tumor growth was monitored via ultrasound imaging. Tumor volumes were calculated using the VEVO-770 software (version 3.0.0, VisualSonics, Inc., Toronto, ON, Canada), and once tumor volume reached 20% of body mass, mice were euthanized and tumor tissue was harvested for subsequent analyses by flow cytometry.

Plasmids used/transfections/transductions

WT transformants stably expressing HuSh PRF1-c-RS shRNAs for Hspa1a and Hspa1b or the shRNA pRFP-c-RS negative control (all purchased from...
OriGene, Rockville, MD, USA) were generated. A retroviral vector encoding p185 Bcr-Abl and GFP (MSCV-IRES-GFP) was used to generate retroviral particles by transfection of Phoenix-Eco cells (Invitrogen).

RNA isolation and quantitative PCR
Total RNA was used to generate cDNA, and quantitative PCR was performed using a 7900HT Fast Real time PCR system (Applied Biosystems, Foster City, CA, USA) using standard conditions for Sybr Green PCR Master Mix (ABI, Life Technologies, Grand Island, NY, USA).

Histopathology and immunohistochemistry
Tumors were first fixed in 4% paraformaldehyde overnight. Those used for paraffin sections were transferred to 10% neutral-buffered formalin and embedded in paraffin wax. 4-μm sections were prepared, stained with hematoxylin and eosin and examined microscopically. Tumors used for cryosections were washed with phosphate-buffered saline after overnight fixation and placed in 30% sucrose at 4 °C before transfer into optimal cutting temperature-embedding compound.

Confocal images
Confocal images were taken using a Zeiss LSM 510 NLO Meta point scanning confocal/multiphoton microscope and Zen software (both from Zeiss Group, Oberkochen, Germany).

Immunoblot
WT E1A/Ras MEFs stably expressing Hspalpha/Hsp10 or negative control RFP-shRNAs were subjected to heat shock for 1 h at 42 °C before recovery at 37 °C for 2 h. Extracts were prepared and analyzed by immunoblotting for HSP70/HSP72 (1:1000; Enzo Life Sciences, Farmington, NY, USA), RFP (1:2000; OriGene) and actin (1:400; MP Biomedicals, Solon, OH, USA).

Culture of Bcr-Abl-transduced BM
Procedures were described as previously.25 WT or Hsp70−/− BM was transduced with Bcr-Abl-GFP, and cells were maintained in culture or introduced into lethally irradiated WT recipients.

Εμ-Myc model of lymphomagenesis
Cohorts of Εμ-Myc mice, WT, heterozygous and null for Hsp70 were monitored for onset of disease.26 Lymphoid organs were harvested for subsequent analysis via immunostaining and flow cytometric analysis.

Migration assays
Migration assays were conducted using the ChemoTx system (Neuro Probe, Inc., Gaithersburg, MD, USA). Splenocytes isolated from mice inoculated with WT or Hsp70−/− E1A/Ras MEF transplants 5 days before were introduced into the upper chamber above WT or Hsp70−/− MEF transplants, and after a 4-h incubation at 37 °C, the cell number in the lower well was enumerated using a Scepter cell counter (EMD Millipore, Billerica, MA, USA).

Antigen cross presentation
Hsp701/3−/− or WT BM was maintained in culture with 20 ng/ml recombinant murine granulocyte macrophages colony-stimulating factor (GM-CSF; Peprotech, Rocky Hill, NJ, USA). After 7 days, enriched dendritic cells (105) were cultured overnight without GM-CSF before addition of Class I OVA peptide 257–264 (100 μM). The following day, OT-1 transgenic T cells were added to the wells, and cells were counted after 72 h.

Statistical analysis
Statistical significance was determined according to the test described in the relevant figure legend.

CONFLICT OF INTEREST
The authors declare no conflict of interest.


Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)