Met induces mammary tumors with diverse histologies and is associated with poor outcome and human basal breast cancer

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Elevated MET receptor tyrosine kinase correlates with poor outcome in breast cancer, yet the reasons for this are poorly understood. We thus generated a transgenic mouse model targeting expression of an oncogenic Met receptor (Met^{mt}) to the mammary epithelium. We show that Met^{mt} induces mammary tumors with multiple phenotypes. These reflect tumor subtypes with gene expression and immunostaining profiles sharing similarities to human basal and luminal breast cancers. Within the basal subtype, Met^{mt} induces tumors with signatures of WNT and epithelial to mesenchymal transition (EMT). Among human breast cancers, MET is primarily elevated in basal and ERBB2-positive subtypes with poor prognosis, and we show that MET, together with EMT marker, SNAIL, are highly predictive of poor prognosis in lymph node-negative patients. By generating a unique mouse model in which the Met receptor tyrosine kinase is expressed in the mammary epithelium, along with the examination of MET expression in human breast cancer, we have established a specific link between MET and basal breast cancer. This work identifies basal breast cancers and, additionally, poor-outcome breast cancers, as those that may benefit from anti-MET receptor therapies.

gene expression profiling | mouse models | epithelial to mesenchymal transition

Breast cancer is a heterogeneous disease that comprises distinct biological entities that are correlated with diverse clinical outcomes and responses to treatment. Gene expression profiling and molecular pathology have revealed that breast cancers naturally divide into the luminal, ERBB2-positive, and basal-like subtypes (1, 2). These subtypes were named to reflect gene expression patterns of the 2 principal cell types of the differentiated breast, luminal epithelial cells lining the duct and lobule, and myoepithelial cells that form a single layer surrounding the luminal cells. The luminal subtype comprises ~60% of breast cancers, is estrogen receptor (ESR1)-positive, and expresses ESR1-responsive genes and luminal markers such as keratin 8/18. Up to 25% of breast cancers are identified with overexpression/amplification of the ERBB2 receptor tyrosine kinase, and these tumors are generally ESR1/progesterone receptor (PGR)-negative. The basal group is characterized as ESR1/PGR/ERBB2-negative and is frequently positive for basal keratins 5/6 (3, 4). Breast cancers within the luminal subtype receive antiestrogen therapies and tend to have a good prognosis. Because of the lack of treatment options, patients within the basal subtype historically have a poor prognosis (1). Hence, an understanding of the signaling pathways active in these tumors is crucial for the generation of targeted therapies.

The MET receptor tyrosine kinase, which is the receptor for hepatocyte growth factor/scatter factor (HGF/SF), is expressed at

elevated levels in 15–20% of human breast cancers (5), and is a prognostic factor for poor outcome (6, 7). High levels of the MET receptor ligand HGF/SF in the serum of breast cancer patients is also correlated with a shorter disease-free interval after surgery (8) and a higher tumor/lymph node/metastasis score (9).

Although several transgenic mouse models have examined the tumorigenic capacity of Met receptor signaling, none have targeted the expression of Met specifically to the mammary epithelium. When either HGF or an activated Met receptor is expressed under the constitutively active metallothionein promoter, transgenic mice develop malignancies, including mammary tumors (10–12). Additionally, multiparous transgenic mice expressing HGF under the mammary-specific whey acidic protein (WAP) promoter develop mammary tumors (13). These transgenic mice highlight the susceptibility of the mammary epithelium to transformation by an enhanced Met/HGF signal.

Here, we describe a murine model of breast cancer generated by the expression of weakly transforming mutants of the Met receptor tyrosine kinase in the mammary epithelium. We demonstrate that Met^{mt} induces mammary tumors with diverse histology, which, based on immunohistochemistry and expression profiling, includes tumors with basal and luminal characteristics. By performing microarray analyses, we reveal that Met^{mt}-induced basal tumors show expression of basal keratins, enrichment for markers of the Wnt pathway, display features indicative of epithelial to mesenchymal transition (EMT), and cluster with human basal breast cancer and murine models of basal-like breast cancer. In human breast cancer, MET shows consistent elevated expression in the basal subtype and identifies patients with poor outcome. Our transgenic mouse model, coupled with human breast cancer data, identifies MET as a rational therapeutic target for both basal and aggressive breast cancer.

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Fig. 1. Met^{mt} mice develop mammary tumors with diverse phenotypes that cluster into 2 groups by microarray analysis. (A) Met^{mt} transgenic mice show a spectrum of histological phenotypes. (Scale bars, 50 μm.) (B) Class discovery analysis of Met^{mt} tumor and normal epithelium segregate samples into 3 main clusters: normal (green), solid tumors (purple), and mixed pathology tumors (red). The significance is calculated by using multiscale bootstrap resampling and is provided for both approximately unbiased (brown) and bootstrap probability (gray) *P* values. The height of the dendrogram represents the distance between cluster centroids.

Results

MMTV/Met^{mt} Induces Tumors with Heterogeneous Histology. We have generated a transgenic mouse model that expresses the Met receptor in mammary epithelium driven by the mouse mammary tumor virus (MMTV) promoter/enhancer. Mice transgenic for wild-type and oncogenic variants of the Met receptor (M1248T, Y1003F/M1248T), hereafter called Met^{mt} (12, 14), develop tumors with moderate penetrance (9–40%) and long latency [381–475 days; supporting information (SI) Table S1] (15). MMTV expression is greatly enhanced during puberty, pregnancy, and involution (16), and, consistent with this, tumor induction was observed predominantly in multiparous mice (Table S1). Within this cohort of mice, ~50% produced carcinomas with solid nodular histopathology (~50%) that are most common to MMTV/Neu and MMTV/PyMT models (16, 17). Interestingly, the remaining 50% induced tumors with either papillary, scirrhous, adenosquamous, or spindle-cell phenotypes (Fig. 1A, Table S2). In general, each animal produced tumors with similar pathology, although a minority of animals produced multiple tumors each with distinct pathology (Table S2).

To confirm that mammary tumors in Met^{mt} mice resulted from integration of the transgene, we first performed PCR analysis on DNA from tumors and matched-normal tissue and demonstrated the presence of the Met transgene in both (Fig. S1A). By using an antibody specific for the transgene, Met^{mt} protein was detected only in tumor tissue and at variable levels, but was undetectable in tumors possessing spindle-cell pathology (5482T, Fig. 1A and Fig. S1B). This is consistent with previous observations demonstrating

suppression of the MMTV promoter in spindle-cell tumors (18). To address the utility of the Met^{mt} transgenic mice as a preclinical model, we examined the ability of epithelial cell lines derived from Met^{mt} tumors to invade (Fig. S1C–F). Using a specific small-molecule inhibitor (PHA-665752) and siRNA for Met, we demonstrate that elevated Akt and Erk1 and -2 activity depends on Met activity (Fig. S1E and F). In addition, the invasive (Fig. S1C and D) capacity of these cell lines also depends on Met, which provides strong support that tumors derived from the Met^{mt} transgenic mice are indeed dependent on Met for a biological response, a criterion required for effective preclinical models.

To better understand the molecular characteristics associated with the distinct histologies found in Met^{mt} tumors, we performed gene expression analyses using Agilent whole-genome mouse arrays. Epithelial tissue from different tumor histologies and matched-normal were analyzed. Unsupervised hierarchical clustering revealed that the arrayed samples naturally fell into 3 distinct classes. The first major division within the data separated all normal samples (“normal epithelium,” green) from tumor samples (Fig. 1B). Within the group of tumor samples, 2 subclusters formed; the first subcluster consisted of tumors with a solid phenotype (“solid,” purple), and the second consisted of tumors with papillary, scirrhous, adenosquamous, and spindle cell pathologies (“mixed-pathology,” red) (Fig. 1B).

To understand the differences between these tumor types, genes differentially expressed between clusters were analyzed for overrepresentation of biological pathways (Tables S3–S8). Genes overexpressed in Met^{mt} mixed-pathology tumors (red, Fig. 1B) compared with the Met^{mt} solid tumors (purple, Fig. 1B) were related to gene ontology (GO) categories involving epithelial and mesenchymal cell differentiation, including genes linked to EMT (Table S3). Overrepresentation of categories involved in tissue remodeling, collagen production, cytokine signaling, cell migration, angiogenesis, inflammatory response, and Wnt, integrin, and TGFβ signaling pathways were also observed (Table S3). In contrast, Met^{mt} solid tumors (purple, Fig. 1B) showed enrichment for GO terms related to apoptosis, cell adhesion, and small GTPase signal transduction (Table S4), supporting the interpretation that distinct tumor pathology is associated with gene expression profiles representing different biological processes. Similar results were obtained by using GSEA and KEGG (Tables S5–S8).

Met^{mt} Mixed-Pathology Tumors Cluster with Basal-Like Murine Mammary Tumors. Transgenic mouse models of breast cancer have recently been analyzed at the gene expression level and were found to correlate with the subtypes defined in human breast tumors (19). To determine whether Met^{mt}-induced tumors resemble a specific subtype of mammary tumors, we used the “866 intrinsic gene set” found to be representative of murine mammary tumors (19). Our samples were combined with this dataset, containing 13 other mouse mammary tumor models, and samples were hierarchically clustered over the intrinsic gene set. This analysis further confirmed that the 2 subtypes of Met^{mt} tumors represent distinct molecularly defined groups (Fig. 2). Met^{mt} solid tumors clustered closely together with mouse models exhibiting solid adenocarcinoma-like pathology, including MMTV/Neu and MMTV/PyMT models (Fig. 2). In contrast, the majority of mixed-pathology Met^{mt} tumors clustered within a group that included p53^{-/-} transplants, p53^{+/-} irradiated (IR), C3(1)/Tag, and WAP/T₁₂₁ tumors, which express basal and mesenchymal related genes (Fig. 2) (19). These models are consistent with our observation that EMT and mesenchymal pathways are enriched in Met^{mt} mixed-pathology tumors (Fig. 4A).

To assess whether Met^{mt} tumors have large-scale transcriptional similarities to human breast cancers, we developed a hierarchical cluster of Met^{mt} tumors with 172 human breast tumors using a cross-species intrinsic gene set (19). The results support the interpretation that Met^{mt} mixed-pathology tumors reflect a basal subtype, because these tumors clustered with human basal breast

Fig. 5. Met^{mt} mixed-pathology tumors show coexpression of Met with basal markers. Immunofluorescence staining of Met (red), basal keratin 5 (purple), and luminal keratin 8/18 (green) in Met^{mt} mixed-pathology and solid tumor controls. Cellular regions exhibiting costaining are represented by pink staining in the merge images. (Scale bars, 20 μ m.)

mixed-pathology Met^{mt} tumors showed coexpression of Met^{mt} transgene with basal marker keratin 5, indicating a correlation between Met transgene expression and basal markers, in contrast to Met^{mt} solid tumors (Fig. 5).

Genes associated with the basal phenotype (3, 4), such as Egfr, Trp63, members of the Wnt pathway, β -catenin (*Ctnnb1*), and transcription factors *Tcf/Lef* (*Tcf4*, *Tcf7*, *Lef1*), were elevated in Met^{mt} mixed-pathology tumors, but not in Met^{mt} solid tumors (Fig. 4 A–C). In serial sections, regions of Trp63 immunopositivity corresponded to regions of Trp53 nuclear staining (Fig. 4B) but were immunonegative in Met^{mt} solid tumors (Fig. 4C). Although human *Trp53* is frequently mutated in basal breast cancer and often associated with high nuclear grade (20), we find no evidence of *Trp53* mutations in our murine model. In support of activation of the Wnt pathway, nuclear localization of β -catenin was observed in Met^{mt} tumors of mixed pathology (Fig. 4B), whereas membranous localization of β -catenin was observed in Met^{mt} solid tumors. Moreover, Met^{mt} mixed-pathology tumors showed elevated expression (*Vim*, *Snail*, *Snai2*, *Acta2*) (Fig. 4A) and immunopositivity of EMT-associated genes (*Vim*, *Acta2*) (Fig. 4B), and these were absent in Met^{mt} solid tumors. Consistent with features found in human basal breast cancers (4), Met^{mt} mixed-pathology tumors were poorly differentiated, a subset showed squamous metaplasia, geographical necrosis, lymphocytic infiltration, high mitotic index, atypical mitotic figures, and nuclear pleomorphism. Together these data demonstrate that Met^{mt} mixed-pathology tumors share histopathological features and basal protein markers consistent with human basal breast cancers. For immunohistochemistry of normal mammary gland control tissues, see Fig. S3.

MET Correlates with Human Basal Breast Cancer and Poor Prognosis.

To establish whether *MET* expression correlates with specific subtypes of human breast cancer, we examined gene expression data obtained from tumor epithelium of human breast cancer patients. Patients included in this dataset were lymph node status positive and negative, and patient characteristics are published in refs. 21 and 22). The 54 human breast cancers within this dataset segregated into 3 main molecular subtypes as defined by immunohistochemistry: luminal (ESR1+ and/or PGR+), ERBB2+ (ERBB2+ and ESR1–/PGR–), and triple-negative (ESR1–/PGR–/ERBB2–) tumors, which reflect the basal subtype. Consistent with these subdivisions, the mean expression of luminal-specific genes *ESR1*, *PGR*, *KRT8*, and *KRT18* were highest among luminal samples and were significantly different among the 3

Fig. 6. A MET gene expression signature clusters tumors primarily of the basal phenotype and correlates with poor outcome. (A) *MET* RNA levels in human breast cancer subtypes. Box-and-whisker plots represent levels of RNA for *MET*, *ESR1*, *KRT6B*, and *ERBB2*. The Kruskal–Wallis rank sum test was used. (B) A Met signature from HGF/Met-stimulated hepatocytes clusters the Netherlands Cancer Institute (NKI) microarray dataset comprising 295 breast cancer patients, into 2 main groups: a MET signature+ cluster (turquoise), and a MET signature– cluster (orange). Breast cancers within the MET signature+ cluster are primarily of the basal subtype ($P = 3.94 \times 10^{-26}$). Color legend refers to the breast cancer subtypes, luminal (blue), ERBB2+ (pink), and basal (red) and to the MET signature status, either positive (turquoise) or negative (orange). (C) Kaplan–Meier analysis based on disease-free survival (DFS) of the patient clusters generated in B ($P = 1.00 \times 10^{-4}$). (D) Kaplan–Meier analysis based on DFS of patients from only the basal subtype, grouped by the clusters generated in B ($P = 3.19 \times 10^{-2}$).

subtypes (Fig. 6A and Fig. S4A). As expected, expression of *ERBB2* was highest among the ERBB2-positive subtype (Fig. 6A). The mean expression of basal-specific markers *KRT6B* and *EGFR* were highest among the basal subtype (Fig. 6A and Fig. S4A). Notably, the mean expression of *MET* was found to be highest in basal samples, with significant differences among the groups ($P = 3.64 \times 10^{-6}$, Fig. 6A). However, when luminal samples were excluded from the analysis, the expression of *MET* was not significantly different between the basal and ERBB2-positive samples ($P = 2.89 \times 10^{-1}$), indicating that *MET* expression may impact both breast cancer subtypes.

To establish whether the MET molecular pathway is activated in a particular subtype of human breast cancer, we examined MET phosphorylation status using antibodies raised to the twin tyrosines within the catalytic loop of the MET kinase (Fig. S4B and C).

Notably, triple-negative breast tumors with high *MET* RNA (Fig. 6A) displayed high MET immunostaining and stained positive with MET phosphospecific antibodies (Fig. S4B), consistent with MET activation and signaling in human basal breast cancers. In contrast, luminal tumors with low *MET* RNA (Fig. 6A) showed no detectable MET or phosphospecific MET immunostaining (Fig. S4C). Moreover, when a MET transcriptional signature (23) was applied to an independent human breast cancer dataset (NKI) (24), the MET signature clearly separated human breast tumors into 2 clusters (Fig. 6B), with the smaller cluster (turquoise) representing an induction of the MET transcriptional response when compared with the larger cluster (orange). Genes expected to be overexpressed in MET activation were significantly higher in the turquoise cluster ($P < 2.20\text{e-}16$), whereas genes expected to be repressed by MET activation were significantly lower in this cluster ($P = 1.90\text{e-}09$). Notably, tumors belonging to the human basal subtype were overrepresented in the turquoise cluster identified by the MET transcriptional signature ($P = 2.99\text{e-}24$), supporting the association of MET with the basal group. The cluster of tumors with a MET-activated transcriptional signature (turquoise) had a significantly worse overall prognosis (Fig. 6C, $P = 1.00\text{e-}04$). Furthermore, the MET signature identified poor outcome within the basal subtype (Fig. 6D, $P = 3.19\text{e-}02$). Unfortunately, there were insufficient luminal and ERBB2-positive samples in the MET-activated group ($n = 3$ and $n = 2$, respectively) to assess whether the MET signature was associated with poor prognosis in these subtypes. In cDNA microarray analyses conducted in a prospectively accrued cohort of women with axillary lymph node-negative (ANN) breast cancer (25), we identified *MET* as differentially expressed between tumors from 48 women who experienced disease recurrence within 4 years of diagnosis when compared with 41 who remained disease-free for >10 years (Table S9; $P = 1.33\text{e-}02$ by standard t test, $P = 8.70\text{e-}03$ by nonparametric Wilcoxon rank-sum test in univariate analysis), an association that was independent of traditional clinicopathological parameters (SI Text, $P = 3.10\text{e-}02$).

To further investigate the correlation between transcriptional activation of *MET* and the basal subtype, we stained for MET protein in a cohort of 668 ANN human breast cancer cases (26). High MET immunostaining was observed in all subtypes, but a significantly greater proportion of basal subtype tumors were MET positive (“high MET,” 65%) as opposed to MET negative (“low MET,” 35%), when compared with the ERBB2-positive and luminal subtypes (Fig. 7A, $P = 6.51\text{e-}03$). Consistent with previous results, the Kaplan–Meier plot demonstrates that MET-positive tumor status correlates with poor disease-free survival (DFS) outcome among ANN patients (Fig. 7A, log-rank $P = 3.94\text{e-}02$). Additionally, MET-positive protein status is associated with poor outcome in univariate analyses irrespective of breast cancer subtype (*SI Text*, $P = 6.31\text{e-}01$ by Cox model test for differences between basal and nonbasal, [Table S10](#) and [Fig. S4D](#)), which is consistent with MET expression being causally related to an aggressive phenotype.

Because we observed a strong induction of EMT genes including Snail (*Snail*) in the murine Met^{mt} mixed-pathology tumors (Fig. 4A) as well as the induction of *SNAIL* (*SNAIL*) mRNA after stimulation of epithelial cells with HGF (Fig. S4E) we investigated coexpression of MET with the downstream EMT target, SNAIL. Notably, tumors exhibiting coexpression of MET with SNAIL showed a significant correlation with poor outcome when compared with those negative for either MET and/or SNAIL (Fig. 7B, log-rank $P = 7.40\text{e-}03$), demonstrating that the combination of MET with an EMT signal, SNAIL, is a strong predictor of poor outcome. This association persisted in multivariate analysis after adjustment for traditional histopathologic prognostic factors (Tables S11 and S12) and for basal/nonbasal status (SI Text and Table S13). Together, these results strongly support a role for MET

Fig. 7. Elevated MET protein correlates with poor-outcome human basal breast tumors and MET/SLNAIL coexpression correlates with poor outcome. (A) Kaplan–Meier analysis shows the relationship between MET protein level and DFS. (B) Kaplan–Meier analysis shows the relationship between MET and SLNAIL proteins and DFS.

signaling both in human basal breast cancers and breast cancers with poor outcome.

Discussion

Understanding the oncogenic pathways that distinguish subsets of human cancer is critical for the development of new therapies. Here, we show that mammary-specific expression of weakly oncogenic forms of the Met receptor promotes the formation of mammary carcinomas with histology, gene expression, and immunohistochemical profiles similar to human basal breast cancers.

Although elevated MET protein levels have been associated with poor outcome in human breast cancer, the role for the MET receptor tyrosine kinase in the induction and development of breast cancer is poorly understood. Mammary-specific expression of MMTV/Met^{mt} surprisingly induced tumors with distinct histological phenotypes (solid and mixed pathology, Fig. 1). The expression profile derived from Met^{mt} mixed-pathology tumors clusters these with human basal breast cancers and murine models of basal tumors (Figs. 2 and 3). This is consistent with findings that Met^{mt} mixed-pathology tumors display histology similar to the human basal subtype and express a series of known basal markers (Fig. 4).

When compared with other murine models, Met^{mt} mixed-pathology tumors clustered with murine tumors having basal characteristics including MMTV/Cre;p53^{+/-}, TgWAP/T121, TgWAP/Tag, and TgC3(1)/Tag tumors (Fig. 2). Within this class, Met^{mt} mixed-pathology tumors shared expression and immunopositivity of EMT-associated genes, including *Tgfb1*, *Snail1*, *Snail2*, and *Twist1*, *Sdc1* (18), in addition to basal-like markers including *Krt 5*, *6a*, *14*, and *17* as well as *Kras* (Figs. 2 and 4). Although a detailed analysis of previous Met/HGF transgenic mouse models is lacking, models with constitutively activated Met, such as Tpr-Met or WAP/HGF (11, 13, 27), also produce mammary tumors with heterogeneous histological phenotypes. In some cases, tumors were shown to be keratin 6-positive (27) with nuclear accumulation of β -catenin (13),

consistent with data observed in our Met^{mt} mixed-pathology tumors (Fig. 4B).

The observation that Met^{mt}-induced tumors with solid pathologies resemble MMTV/Neu, MMTV/PyMT, and MMTV/ras tumors within a luminal subtype (Figs. 2 and 4A) may reflect the ability of the Met^{mt} carrying the Y1003F mutation to promote enhanced activity of the ras pathway (14). The presence of both luminal and basal-like tumor subtypes raises the possibility that Met may target a multipotent progenitor cell(s) with the capacity to differentiate toward each lineage. In support of this, coexpression of Met with basal and luminal markers, keratin 5 and 8/18 respectively, was observed in cells within Met^{mt} mixed-pathology basal-like tumors but not in solid luminal-like tumors (Fig. 5). Moreover, cells immunopositive for nuclear Trp63 or Trp53 in Met^{mt} basal-like tumors (Fig. 4B) may be indicative of a stem/progenitor cell population (28).

We show that elevated MET RNA, protein, and transcriptional response are associated with the human basal subtype (Figs. 6 and 7A) and that when elevated, MET immunopositive sections stain with MET phosphospecific sera indicative of MET activation (Fig. S4 B and C). Moreover, in patients who are lymph node-negative at presentation, elevated MET protein is associated with poor outcome (Fig. 7A). Specifically, in the node-negative cohort, elevated MET protein is significantly correlated with the basal subtype ($P = 6.51e-03$, Fig. 7A). Among the biological processes highlighted in gene expression studies with the Met^{mt} basal tumors are genes involved in EMT (Fig. 4A and Table S3). Within EMT-related genes, one of the most differentially regulated genes in Met^{mt} mixed-pathology tumors is the *Snail* transcription factor, which regulates EMT during development. Strikingly, elevated levels of both MET and SNAIL in tumors is significantly correlated with poor outcome in lymph node-negative patients when compared with either protein alone (log-rank $P = 7.40e-03$ and Fig. 7B).

This is a first report of an MMTV-driven receptor tyrosine kinase mouse model that produces tumors resembling human basal breast cancer. Significantly, an adjoining manuscript demonstrates a similar basal phenotype induced by a different oncogenically activated

Met receptor tyrosine kinase driven from its endogenous promoter (29). Together, this provides strong support that activation of Met signaling pathways play an important role in the induction of tumors of the basal subtype. Because human basal breast cancer is especially difficult to treat due to a lack of understanding of the genes and processes involved in its induction, developing a model for the dissection of this subtype is of utmost importance. Here, we establish that the MET receptor is casually associated with basal and poor outcome breast cancer. Elevated MET RNA, protein and a MET transcriptional profile correlates with human basal breast cancer indicating that MET may prove a useful therapeutic target in the management of these patients.

Methods

Transgenic Mice. MMTV/Met mice were generated as described (15). The 8.7-kb fragment containing the Met cDNA, MMTV promoter, and SV40 polyA was injected into the pronuclei of FVB/N zygotes and implanted into FVB/N hosts by the McIntyre Transgenic Core Facility (Montreal, Canada). Mice were housed in accordance with McGill University Animal Ethics Committee guidelines.

Mouse and Human Tissue Processing. Processing of human and mouse tissues have been described (21, 30). For human ANN breast cancer cases, a prospectively ascertained consecutive series was enrolled, as previously described (25). Consent was obtained from patients in this Institutional Review Board-approved study.

Immunohistochemical Analysis. Tissue sections were stained by using the ABC Elite and MOM reagents (Vector Laboratories). Detailed procedures are provided in *SI Text*.

Gene Expression Microarray Data. RNA was prepared for hybridization, and data were analyzed as previously described (21). Detailed procedures are provided in *SI Text*.

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