

Mndal, a new interferon-inducible family member, is highly polymorphic, suppresses cell growth, and may modify plasmacytoma susceptibility

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The human HIN-200 gene cluster and its mouse counterpart, the interferon inducible-200 (*Ifi200*) family, both on Chr 1, are associated with several diseases, including solid tumors and lupus. Our study was initiated to identify the modifier gene(s) encoded by the *Pctm* locus, in which mouse B-cell plasmacytomas induced by pristane are associated with heterozygosity of Chr 1 genes near the *Ifi200* cluster. A screen for differentially expressed genes in granulomatous tis-

suces induced by pristane in resistant and susceptible strains identified a new *Ifi200* member whose expression was 1000-fold higher in the strain carrying the resistant allele of *Pctm* and was the most highly expressed *Ifi200* gene. The gene, designated *Mndal* (for MNDA-like, myeloid nuclear differentiation antigen-like), was absent in the susceptible genome, as were genomic sequences upstream of *Ifi203*, the gene adjacent to *Mndal*. Ectopic expression of *MNDAL* suppressed

cell growth, which, together with the disease susceptibility of heterozygotes at the *Pctm* locus, suggests that *Mndal*, perhaps with *Ifi203*, acts as a tumor suppressor and display(s) haploinsufficiency. *Mndal* is highly polymorphic among inbred mouse strains, because it is absent in 10 of 24 strains. This polymorphism may have implications for other disease modifiers mapping to the same region. (Blood. 2009;114:2952-2960)

Introduction

B-cell tumor development is a complex genetic trait controlled by multiple low penetrance susceptibility genes modified by macro/microenvironmental influences. The pristane-induced mouse plasmacytoma (PCT) system represents an experimental tumor model, because multiple genes regulate susceptibility to PCT.¹ Pristane (2, 6, 10, 14-tetramethylpentadecane [TMPD]), produced by zooplankton and found in high concentrations (in micrograms per gram) in some fish species,² is used in the production of monoclonal antibodies and can induce a lupus-like disease in mice.³ In the PCT system, intraperitoneal TMPD injection induces chronic inflammation within 14 to 21 days and produces mesenteric oil granulomas in response to cytokines elicited from TMPD-containing macrophages.^{4,5} TMPD has recently been shown to elicit type I interferon through a TLR7-dependent pathway.³

In BALB/cAnPt(BALB) mice, PCTs develop in 60% of TMPD-treated mice, whereas in DBA/2N(DBA) mice, they remain tumor free (0%) for longer than a year.^{6,7} Resistance to PCTs is genetically dominant; however, 2% of (BALB×DBA)F1 hybrids develop tumors with a latency period longer than a year.¹ Tumor susceptibility is less penetrant in NZB/BINJ compared with BALB; only 30% of NZB/BINJ mice develop PCTs.^{6,7} Different numbers of susceptibility loci in the strains probably to contribute to the difference in incidence. It should be noted that any given strain is likely to carry both tumor resistance and susceptibility alleles, and it is the relative effect of these genetic alleles that will determine the degree to which the strain is phenotypically resistant or susceptible.

Plasmacytoma susceptibility is linked to homozygosity of BALB(C/C) alleles of genes on mouse Chr 4 as determined by a linkage study involving the backcross BALB×(BALB × DBA)F1.^{1,8}

This genetic cross, in conjunction with development of congenic lines,⁸ led to identification of loci, designated *Pctr* or *Pctm*, that contribute to PCT susceptibility.^{1,9-11} Genes identified for *Pctr1* and *Pctr2*, respectively, are, *Cdkn2a* (p16), identified by a candidate gene approach, and *Frap* (mTOR), by positional cloning.⁹⁻¹¹ The BALB alleles of both p16 and mTOR encode efficiency alleles, whose functional activities are much less active than the DBA allele.⁹⁻¹¹ PCT-susceptible NZB/BINJ mice carry the same high-efficiency allele of p16 as that of DBA¹⁰ and, as such, carry a PCT-resistance allele at *Pctr1* despite their 30% incidence of PCTs. In contrast, NZB mice carry a PCT-susceptibility allele at *Pctr2* because its mTOR allele is the less efficient BALB allele,⁹ defective in phosphorylating p53.

Although BALB is the most PCT-susceptible strain, not all of its potential modifier genes encode susceptibility alleles. Similarly, the resistance of DBA to PCTs does not mean that it only carries resistance alleles. In the cross between BALB and DBA that led to identification of p16 and mTOR as *Pctr* loci, a locus on Chr 1 was also detected¹ whereby DBA (D) is predicted to have a susceptibility allele and BALB (C) a resistance allele. This PCT modifier of resistance/susceptibility (*Pctm*), linked to Chr 1 near the *Fcgr2* locus, was the only susceptibility locus in the backcross linked to heterozygosity (C/D alleles), suggesting that DBA carries a susceptibility gene for plasmacytomagenesis on Chr 1 that displays a susceptible phenotype when *Pctm* is heterozygous; these same progeny were C/C at p16 and mTOR. The interval containing the *Pctm* locus also includes the hematopoietic interferon-inducible 200 (*Ifi200*) gene cluster, whose human counterpart is *HIN-200*. This family, consisting of a series of related genes sharing various

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degrees of homology, has been linked to modifiers for several diseases, including systemic lupus¹² and solid tumors.¹³⁻¹⁵ Malignancy has also been associated with somatic mutation and epigenetic silencing of alleles within these clusters.¹⁶

In this study, gene expression profiling identified a new member of the *Ifi200* cluster, which shares homology with human *MNDA* (myeloid nuclear differentiation antigen) and whose level of expression was found to have the greatest difference in inflammatory tissues between BALB and DBA mice. We have designated the murine gene *Mndal* (myeloid nuclear differentiation antigen-like), and it maps to the region of mouse Chr 1 also implicated as the site of the *Pctm* susceptibility locus in DBA. Our analysis indicates that *Mndal* is expressed in BALB, whereas its entire open reading frame is absent from DBA.

Methods

Gene expression

With the use of animal protocols approved by the Institutional Animal Care and Use Committee of the National Institutes of Health (NIH), mice were given 0.5 mL TMPD intraperitoneally. Three and 18 days later, total RNA was extracted from mesentery and spleen of experimental/control animals, with the use of TRIzol (Invitrogen). Labeled aRNA prepared from 1 μ g RNA (MessageAmpII aRNA Amplification kit; Ambion) was hybridized to mouse genome 430 2.0 array chips, processed on Workstation 450, and analyzed with Gene Chip Operating Software (Affymetrix). Differential expression was assessed by analysis of variance (Partek Genomics Suite). Expression levels (average difference; GeneChip 3.1) are presented for probe sets targeting EST sequences that overlap the *Mndal* full-length cDNA. Reverse transcription–polymerase chain reaction (RT-PCR) methods are described in the supplemental Methods section (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

cDNA cloning

To identify, clone, and sequence the most differentially expressed gene, target sequences of 2 probe sets (Affy 1426906AT [labeled as *Mnda-like*] and 1452231xAT) were used to search the National Center for Biotechnology Information (NCBI) expressed sequence tag (EST) database.¹⁷ EST sequences were identified and used to build a virtual transcript of *Mndal*. A cDNA clone recognizing one of the ESTs, BE68641, was obtained from Invitrogen, and the full-length double-strand sequence analyzed contained an 1829-base pair (bp) cDNA from a C57BL/6 mouse thymus; it includes the entire coding sequence, 5' and 3' untranslated regions, and polyA tail. First-strand cDNA was synthesized with polyA RNA from BALB spleen (SuperScriptIII first-strand kit; Invitrogen). PCR amplification was performed with *Mndal*-specific primers 1, 2 (supplemental Table 1). Plasmid construction, genomic sequence analysis, and genetic linkage analyses are all described in supplemental Table 1.

Cellular localization assay

Cells grown on coverslips were transiently transfected with V5-His–tagged *Mndal*, V5-His–tagged *Ifi205*, or control plasmid, with the use of Lipofectamine (Invitrogen) or AMAXA Buffer 5 with a Nucleofector II. Cells were fixed with 3.5% paraformaldehyde 24 hours after transfection, permeabilized with 0.5% Triton X-100, blocked with 10% fetal bovine serum (FBS), and blotted with anti-V5-fluorescein isothiocyanate (FITC) antibody (Invitrogen). Stained cells were mounted with Vectashield with 4',6'-diamidino-2-phenylindole (DAPI), and examined using a fluorescence microscope. Western blot methods are described in supplemental Methods.

Interferon treatment of cell lines

Cells were seeded in 100-mm plates and grown for 24 hours in Dulbecco modified Eagle medium with 10% FBS. At 90% confluence, cells were treated with or without interferon- α at 1000 units/mL for 18 hours. Total RNA was prepared with RNeasy mini kit (QIAGEN), and expression of *Ifi200* genes was examined by RT-PCR.

NIH3T3 colony-forming assay

Cells were grown in Dulbecco modified Eagle medium, 10% FBS at 37°C, and 5% CO₂. For the colony-formation assay, 300 ng of either pEV-15 or pEV19-Mndal or *Ifi-203* plasmid was cotransfected with 50 ng pSV2Neo into 3 \times 10⁵ NIH3T3 cells in 35-mm plates with the use of calcium phosphate precipitation. Cells were split into five 100-mm plates 24 hours after transfection. Colonies of transfected cells were selected by growing in medium with G418 at a concentration of 0.5 mg/mL. Colonies were counted 2 weeks after selection, and efficiency of colony formation was determined by comparing the number of *Mndal*- or *Ifi203*-transfected colonies with those of empty vector–transfected cells. Eight independent experiments were performed with at least 2 different plasmid preparations for control *Mndal* and *Ifi203* plasmids.

Supplemental data

Materials provided online include details about the analysis of the genomic structure of *Mndal*, including the 30 primer sequences used in PCRs (supplemental Table 1). Alignments of EST sequences used to identify and clone *Mndal* are presented (supplemental Figure 1). Sequence comparisons of *Mndal* between C57BL/6J and BALB/cAnPt are made (supplemental Figure 2). GEP datasets at GEO/NCBI were mined for *Mndal* expression in B-cell populations. Supplemental Figure 4 shows *Mndal* expression from a NP-CGG immunization study,¹⁸ and in B and plasma cell tumors from XBP1s transgenic mice.¹⁹ The alignment of the HIN “a” domains of mouse IFI205, MNDAL, and human MNDA and AIM2 proteins are also presented (supplemental Figure 3). Detailed methods for RT-PCR, genomic organization, and linkage analysis; Western blotting; and isolation of cell populations are provided online.

Results

Gene discovery by microarray analyses

Gene expression profiling was used to identify genes differentially expressed between BALB and DBA strains, by comparing profiles from the pristane-primed mesenteries of mice and searching for differentially expressed genes mapping to Chr 1. Microarray analysis was performed with RNA isolated from at least 2 mesenteries and spleens from each strain, as well as RNA from 2 to 3 different samples of each of 4 BALB PCT cell lines, using the whole-genome Affymetrix mouse chip. Affy probe set 1426906_AT (originally annotated as *Ifi205-like*) was the most differentially expressed gene, with greater than 1000-fold difference between the 2 strains (Figure 1). This probe set gave a strong signal in the mesenteries of BALB mice and, furthermore, mapped to Chr 1 at position 175 694 010 within the *Pctm* interval. The same oligopair, 1426906_AT, was not expressed in DBA, as scored by Gene Chip Operating Software analysis. Another differentially expressed gene mapping to the *Pctm* region was *Ifi203*, at position 175 850 538; its expression in BALB mesenteries was 19-fold higher than DBA. Reevaluation of 1426906_AT by RT-PCR confirmed that it was not expressed in DBA but was highly expressed in BALB; *Ifi203* was also more highly expressed in BALB than in DBA, but the difference was approximately 3- to 4-fold (Figure 2A). The microarray data are available in the GEO public database under accession no. GSE17297.

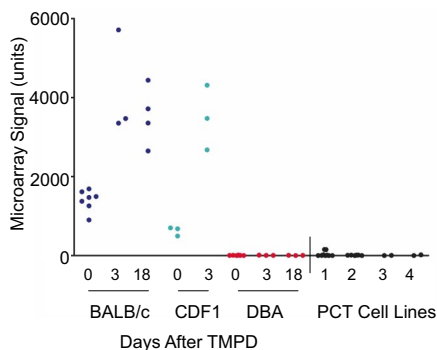


Figure 1. Differential expression of *Mndal* (Affy 1426906_AT) in BALB versus DBA before and after TMPD treatment. The signals from individual RNA samples from the mesenteries of BALB (blue), BALB × DBA F1 hybrids (CDF1; green), DBA (red), and (PCT) cell lines (black) are shown.

Clones and sequence analysis of *Mndal*

Analysis of the region surrounding Affy 1426906_AT determined that this region is highly repetitive; direct cloning and sequencing of several BALB and DBA genomic fragments was required to characterize the gene recognized by the probe set (GenBank bankit 1163875, 1165658, 1165663, 1165660). Sequence analysis showed that 1426906_AT targets a sequence that does not match any previously reported gene in the database.

To identify the gene, the 193-bp target sequence of 1426906_AT was used to search the dbEST database.¹⁷ Several EST clones were identified that matched the 1426906_AT sequence (supplemental Figure 1). A 459-bp EST, BE686461A, was recognized by Invitrogen clone (3377079) isolated from a C57BL/6J (B6) mouse thymus cDNA library, and our sequence data showed it contains an 1839-bp cDNA insert, including the 3' end polyA tail (GenBank

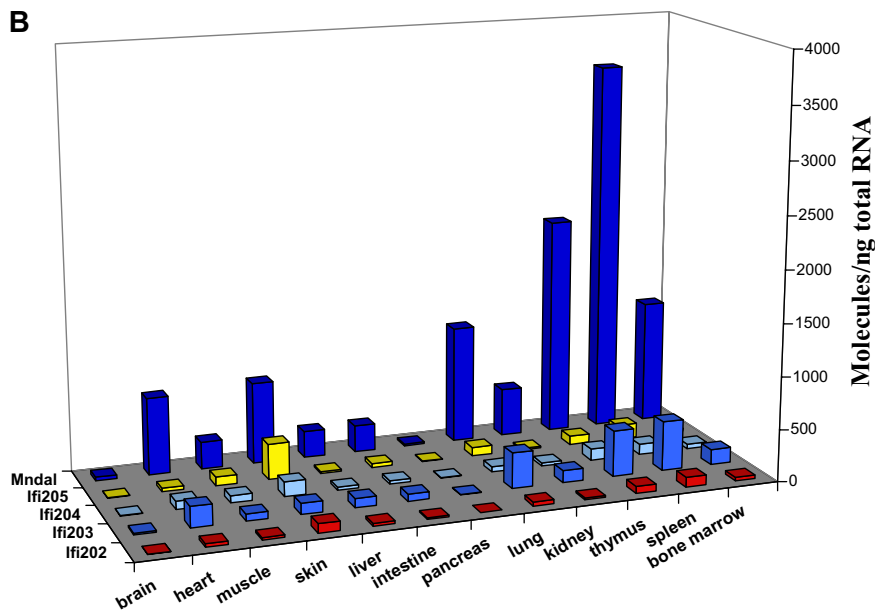
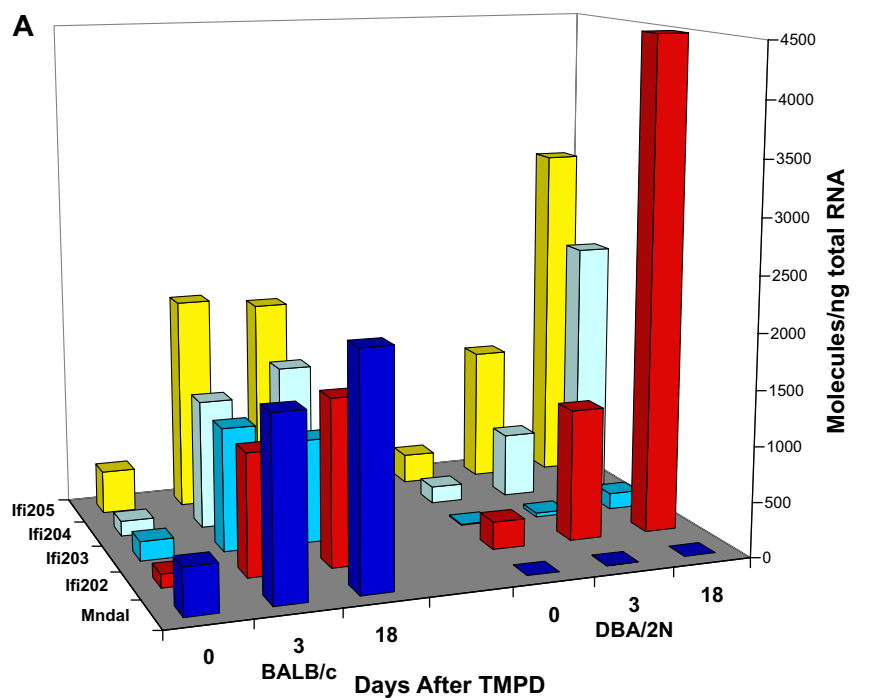


Figure 2. Expression of *Ifi200* genes. (A) Expression of *Ifi200* genes in the mesenteries of BALB and DBA mice at days 0, 3, and 18 after TMPD. *Mndal* (dark blue) was not expressed in DBA; similarly low levels of *Ifi203* (medium blue) were also seen in DBA relative to BALB at all time points. *Ifi202* (red) and *Ifi205* (yellow) levels were higher in day 18 tissues in DBA relative to BALB. (B) Tissue-specific expression of *Ifi200* genes in untreated adult BALB mice. *Mndal* expression (dark blue) is higher in BALB tissues than that of other *Ifi* genes. Splenic and thymic tissues showed the highest levels of expression. Other bars represent expression levels of *Ifi202* (red), *Ifi203* (medium blue), *Ifi204* (light blue), and *Ifi205* (yellow).

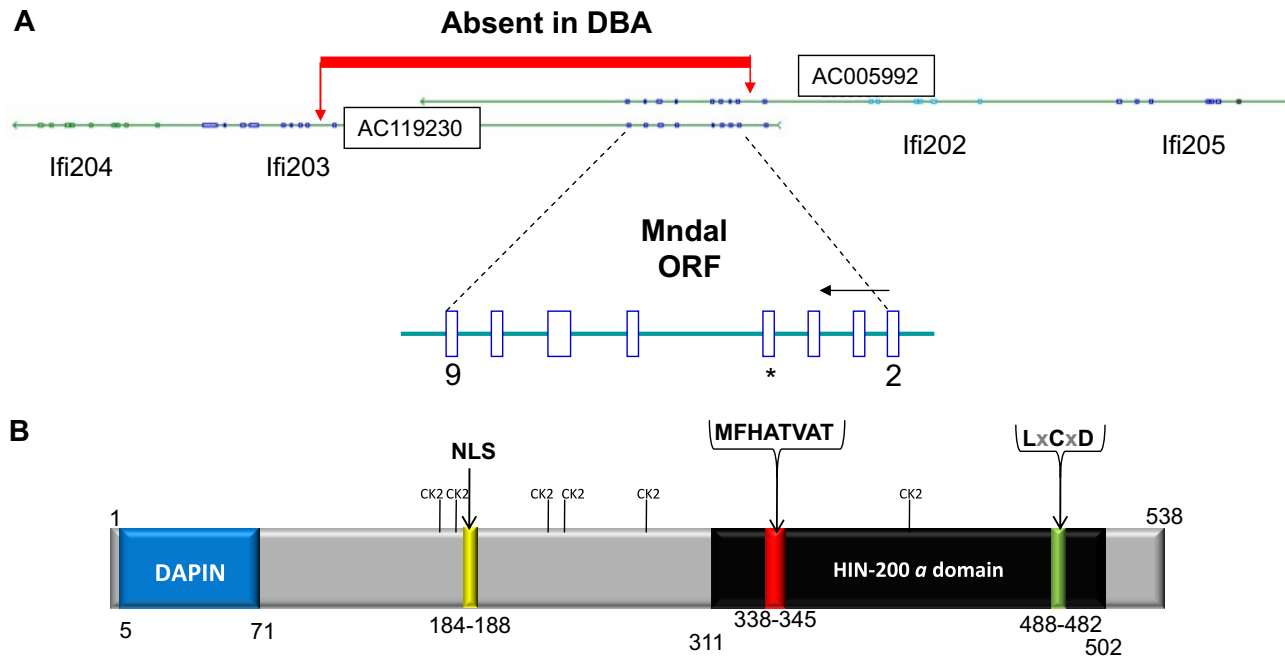


Figure 3. Protein and gene structure for *Mndal*. (A) Two BAC clones harbor *Mndal* sequences. BAC clones are oriented to show gene order on Chr 1 from centromere to telomere. The ORF of *Mndal* starts at exon 2 and finishes in exon 9. Sequence analysis of genomic DNA showed a segment absent in DBA from within intron 1 of *Ifi203* to intron 2 of *Mndal*. *Exon 5 harbors sequences unique to *Mndal*. (B) Predicted structural domains of MNDAL. The canonical HIN “a” domain is highlighted in black and contains the highly conserved MFHATVAT motif thought to mediate dimerization and protein-protein interactions. The protein also contains a nuclear localization signal (NLS), a possible Rb-binding site (LxCxD), and several weak (S/T-P) Cdk2 sites.

bankit 1165668). The target sequence of 1426906_AT matched the cDNA sequence isolated from the B6 thymus 100% near the 3' end (supplemental Figure 1).

Further analysis showed the cDNA is from a novel gene with high sequence homology to members of the human *HIN-200* and murine *Ifi200* families. We designated the gene as *Mndal* (Mnda-like), because of its high degree of homology with human *MNDA*. The full-length *Mndal* cDNA was also isolated and sequenced (GenBank bankit 1156570) from the spleen of BALB/cAn mice. We found that the first 2 exons of the *Mndal* open reading frame (ORF) are 100% identical to exons 1 to 2 of *Ifi203* (exons 1 of *Mndal* and *Ifi203* are both noncoding), and the last 4 exons of *Mndal* are 97% to 99% identical to the last 4 exons of *Ifi204* and *Ifi205*; these exons encode the 200 amino acid “a” domain. Only one exon in the ORF had sequences totally unique to *Mndal* (Figure 3A). By virtue of its chromosomal location and homology to *Ifi* genes, *Mndal* is a member of the *Ifi200* family, whereas the unique exon verifies it is a new gene.

Gene structure and genomic location

By screening the NCBI database¹⁷ with *Mndal* sequence, 2 overlapping mouse BAC clones (AC005992, AC119230) were identified that contained sequences encompassing the *Mndal* locus (Figure 3A). The structure of the *Mndal* gene was determined by comparing cDNA and BAC clone sequences and confirmed by PCR and sequencing of genomic DNA from BALB. The resulting gene structure, with its ORF of 1614 bp predicting a putative protein of 538 amino acids (Figure 3B) distributed over 8 exons (exons 2-9), is consistent with that predicted by Mapview software and Murine Genome Build 33-35 (Figure 3A; supplemental Figure 1). The ORF spans approximately 23 kb and is located in the distal area of chromosome 1 in the *Ifi200* cluster, between *Ifi202* and *Ifi203* (Figure 3A).

The *Ifi200* cluster lies within the same interval, *DIMit16* to *Mtv7*, as the *Pctm* locus.¹ The estimated distance between *DIMit16* and *Mtv7* is 8.8 cM and corresponds to 8.7 Mb in the current build of the mouse genome map (NCBI). Analyses of 163 backcross progeny for an additional 4 *DIMit* markers lying on either side of *Fcgr2* continue to support linkage of *Pctm* to each of these markers with the same probability ($P < .005$). The *Pctm* tumor susceptibility phenotype still has the fewest recombinants with *Fcgr2* (C/D) genotypes. *DIMit 206* has only 1 additional recombinant from that observed for *Fcgr2* (supplemental Figure 2). Analysis of the recombinants suggests that *Pctm* lies between *DIMit145* and *DIMit206*. These data would estimate an interval of approximately 5.2 Mb.

Genetic polymorphism of *Mndal* among mouse strains

To understand what accounts for the lack of *Mndal* expression in the DBA mouse, we compared the genomic structure of *Mndal* and its neighboring sequences between BALB and DBA. Genomic DNA from the kidney of DBA mice was used for PCR and sequence analysis. When the sequence obtained (GenBank bankit 1165663, 1165660) was aligned with that of the BALB strain, it indicated that the *Mndal* gene is absent in the DBA mouse (Figure 3A). Sequence data also showed that the genomic segment between *Mndal* and intron 1 of *Ifi203* is also missing in DBA.

To determine the extent to which *Mndal* is conserved or absent in mouse strains, genomic DNA from 24 inbred strains was examined with 2 sets of allele-specific primers for *Mndal* (Table 1; supplemental Table 1). Some pairs of these strains are known to differ in their responses to cancer induction or autoimmunity, with the modifier gene in question mapping to the region of Chr 1 that includes *Mndal*. Fourteen strains show the expected PCR product that indicates the presence of *Mndal*. However, the gene is missing in the other 10 inbred strains, indicating that its absence represents a frequent polymorphism. In addition, comparison of *Mndal*

Table 1. Presence versus absence of *Mndal* gene in mouse strains and cell lines

Mouse strain, BAC clone, and cell line distribution pattern	
BALB/cAnPt variant (<i>Mndal</i> present)	
Mouse strains	BALB/cJ, BALB/cHeA, CBA/N, C57L/J, C57BL/6J, C57BL/10/SnJ, 129/J, C3H/HeJ, C3H/HeN, NOD, NZW/LacJ, <i>Mus castaneus</i> , <i>Mus molossinus</i> , <i>Mus spretus</i>
BAC clones	AC005992 (129X1/SvJ), AC119230 (C57BL/6J)
Cell lines	BALB3T3 fibroblasts, XRPC24 PCT (BALB-derived), TEPC1165 PCT (BALB-derived)
DBA/2NPt variant (<i>Mndal</i> absent)	
Mouse strains	AKR/N, A/J, DBA/2J, NON, NZB/BINJ, P/J, SENCARA/Pt, SWR/J, STS/A, <i>Mus caroli</i>
BAC clone	AC006944(129S6/SvEvTac)
Cell lines	NIH3T3 fibroblasts, D210–403 PCT (DBA-derived), D281–288 PCT (DBA-derived)

The presence/absence of the *Mndal* gene was based on the presence of PCR products using two different primer pairs (supplemental Table 1). Determination that 129S6/SvEvTac has the same structure as that of DBA comes from sequence analysis of BAC clone AC006944.

sequences from 2 strains with an intact *Mndal* gene, BALB and B6, showed several single nucleotide polymorphisms, resulting in amino acid changes in MNDAL between the 2 strains (supplemental Figure 2). This comparison suggests that *Mndal* sequences among the inbred mouse strains harboring the gene are probably polymorphic.

Ifi200 mRNA expression levels in BALB versus DBA

The mRNA expression of *Mndal* and other *Ifi200* family members was compared in BALB and DBA by RT-PCR in the mesentery, with and without TMPD (Figure 2A), and in other tissues from BALB (Figure 2B). Mesentery from unprimed BALB mice contained approximately 200 copies of *Mndal* mRNA per 1 ng total RNA. This value increased approximately 5-fold after 3 days of TMPD treatment and was still at that level 15 days later (Figure 2A). The levels of *Mndal* also increased in the lung but decreased in spleen in response to TMPD (data not shown). As noted in Figure 1, there was no detectable signal of *Mndal* in the mesenteries of DBA mice. In BALB, basal levels of *Ifi202*, *Ifi203*, *Ifi204*, and *Ifi205* were detectable in the mesentery and were induced further by TMPD (Figure 2A). In DBA, this was also true for *Ifi202*, *Ifi204*, and *Ifi205*, although the response appeared to be slower than in BALB. By contrast, *Ifi203* was not detectably expressed in untreated mesenteries of DBA mice, and TMPD treatment led only to its low-level expression by day 18 (Figure 2A). This result correlates with the absence of 5' sequences in *Ifi203* noted in Figure 3, indicating possible impairment of the DBA *Ifi203* promoter. Another possibility is that promoter sequences used by *Mndal*, which are present in DBA, may be driving expression of the *Ifi203* gene. In either case, the induction of *Ifi203* expression is lower in DBA than in BALB. The low-to-absent expression of *Mndal* and *Ifi203* in DBA suggests that the defect in either or both genes could contribute to the plasmacytomagenesis associated with *Pctm*.

Examination of basal mRNA expression in multiple tissues of BALB mice showed that *Mndal* expression is tissue specific and that it is the most highly expressed *Ifi200* gene in BALB tissues

(Figure 2B). The highest levels of *Mndal* were observed in spleen and thymus (2000 copies/ng total RNA). Bone marrow, lung, skin, and heart expressed more moderate levels (approximately 1000 copies/ng total RNA), whereas low levels were seen in muscle, liver, and intestine. The other *Ifi200* genes were expressed in a similar pattern, but all at a lower level; as with *Mndal*, brain and pancreas had little or no expression of the other *Ifi200* genes. Although spleen and thymus express all members of the family, *Mndal* is clearly the predominant gene, because it is expressed at levels at least 5 times higher than *Ifi203* and 20 to 30 times higher than *Ifi202*, *Ifi204*, and *Ifi205* (Figure 2B). These results suggest that *Mndal* may be controlled by upstream signals distinguishable from those controlling some of the other *Ifi200* genes and/or that the *Mndal* promoter is more sensitive to the same signals.

Expression in B cells

CD19⁺ B cells, isolated from control versus 18-day pristane-primed mesenteric tissues, showed consistent increases in *Mndal* expression after pristane. *Mndal* expression, as assessed by RT-PCR increased after pristane approximately 3-fold (2021 vs 5859 molecules/ng RNA) in splenic B cells, approximately 5-fold (993 vs 4784 molecules/ng RNA) in bone marrow cells, and 103-fold (14 vs 1451 molecules/ng RNA) in mesenteric B cells. *Mndal* expression also increased 1.5-fold (2772 vs 4282 molecules/ng RNA) in splenic T cells. Relatively high levels of *Mndal* were also found in mesenteric macrophages (3522 molecules/ng RNA). In addition to our experimental data, NCBI GEO datasets containing microarray data for sorted B cells were also mined for *Mndal* expression. A time-course study involving NP-CGG immunization of C57BL/Ka (a strain capable of sustaining myeloma bone lesions) mice showed high levels of *Mndal* in naive (nonimmunized B220⁺) B cells and (immunized 10 weeks) memory B cells.¹⁸ In contrast, *Mndal* expression was modest in plasma cells (immunized 1 week) and even lower to very little expression in germinal center B cells (immunized 2 weeks¹⁸; supplemental Figure 4A).

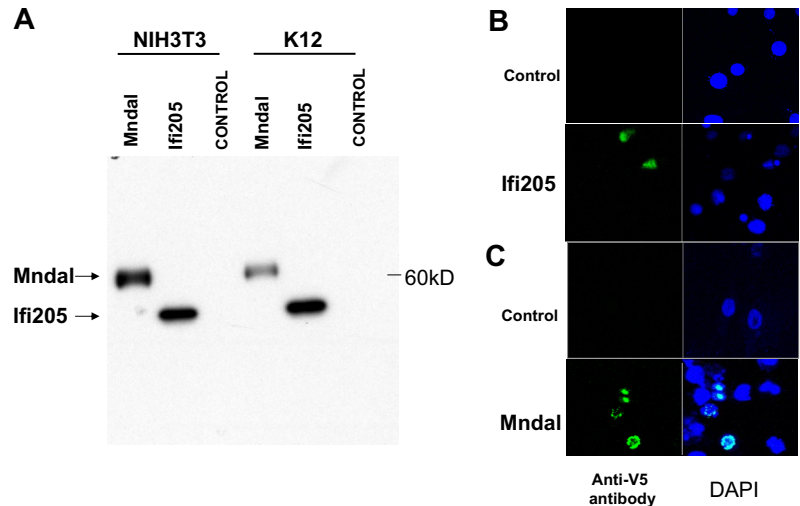
Expression in plasma cell tumors

As expected, the 2 PCT cell lines derived from DBA mice (Table 1; Figure 1 lane 3, D2107-403; lane 4, D281-288) did not express *Mndal*, because the gene is absent from the DBA genome. Unexpectedly, there was also no detectable signal in the BALB-derived PCT cell lines (Table 1; Figure 1A lane 1, XRPC24; lane 2, TEPC1165). The absence of signal in the BALB PCT cell lines is not due to deletion, because the gene remained intact in the lines (Table 1). A similar observation was made in a study of myeloma tumors arising in XBP1s mice in which gene expression of *Mndal* was lower in the tumors than in XBP1s B cells¹⁹ (supplemental Figure 4B).

Protein structure and cellular localization

MNDAL contains a conserved 200–amino acid motif at its C-terminus referred to as the “a domain” (Figure 3B). MNDAL also has a conserved N-terminal basic region, including a DAPIN (Domain in APOptosis and Interferon Response) motif, a consensus nuclear localization signal, a MFHATVAT motif thought to be important in mediating dimerization and protein-protein interactions, a potential Rb-binding site (LxCxD), and several weak (S/T-P) Cdk2 binding sites. Sequence alignment showed that MNDAL is most closely aligned with IFI205, with which it shares 55% identity in full-length and 86% identity in the “a” domain. Compared with the human *HIN-200* genes, MNDAL is most

Figure 4. MNDAL protein expression. (A) Western blot analysis of cells transfected with V5-His–tagged IFI205 (detected at ~ 50 kDa) and MNDAL (detected at ~ 60 kDa). (B-C) Immunofluorescent staining of NIH3T3 cells transfected with V5-His–tagged IFI205 (B) and XRPC24 PCT cells transfected with V5-His–tagged MNDAL (C). Staining with V5 antibody (green) showed overlap with DAPI (blue) stained nuclei. Cells were examined at 25°C with a Zeiss LSM 510 NLO confocal system (Carl Zeiss Inc) with an Axiovert 200M inverted microscope and operating with a 2-photon laser tuned to 750 nm, 25 mW argon laser tuned to 488 nm, and 1 mW HeNe laser tuned to 543 nm. Cells were imaged with a 63×/1.4 NA Zeiss Plan-Apochromat oil-immersion objective. Digital images (512 × 512 pixels, 8 bit) were collected with the use of the Zeiss AIM software with a scan zoom from 2 to 4 and a multitrack configuration in which the FITC, Cy3, and DAPI signals were collected sequentially with a BP 500- to 530-nm filter, with BP 565- to 615-nm filter, and BP 390- to 465-nm filter after excitation with 488-nm, 543-nm, and 750-nm laser lines, respectively.



homologous to MNDA, sharing 25% and 57% identity to the full-length and “a” domain alignment, respectively (supplemental Figure 3). MNDAL also shares 22% and 42% identity with full-length and “a” domain AIM2 sequences, respectively (supplemental Figure 3).

To confirm the production of MNDAL protein from its transcript, the *Mndal* ORF was cloned into the pcDNA3/V5His vector in-frame with the V5-His tag. When the resulting plasmid was transfected into NIH3T3 and mouse K12 osteosarcoma cells, it expressed a single band of approximately 60 kDa, which was larger than the V5-His–tagged IFI205 control (Figure 4A). To examine the subcellular location of MNDAL, V5-His–tagged *Mndal* was expressed in NIH3T3 and mouse XRPC24 PCT cells by transient transfection. V5-His–tagged *Ifi205* was also transfected into NIH3T3 cells, as a control. The proteins encoded by other members of the *Ifi200* family have a predominantly nuclear localization.²⁰ As expected, IFI205 protein was localized to the nucleus (Figure 4B), and a similar localization was also found for MNDAL (Figure 4C, data shown for XRPC24).

Interferon induction of the *Ifi200* genes in vitro

To examine whether the expression of endogenous *Mndal* is induced by interferon, NIH3T3 and BALB3T3 cell lines were treated with interferon- α . In BALB3T3 cells, which harbor the intact gene, this treatment induced higher levels of *Mndal* expression than in untreated cells, and a similar response was seen with the other Ifi genes examined (Figure 5A). By contrast, *Mndal* was not induced in NIH3T3 cells, which correlated with its absence in those cells (Figure 5B). As controls, interferon treatment of NIH3T3 did induce other Ifi genes. *Mndal* expression was also shown to increase in isolated B cells stimulated with *Ifn* β ²¹ (Figure 5C NCBI GEO). This same study shows that active influenza infection results in a 2-fold increase in *Mndal* expression in lymph node B cells, in wild-type mice, but no change in B cells from interferon receptor (*Ifnr*) null mice²¹ (Figure 5C).

Growth inhibition

Previous studies have suggested that other *Ifi200* genes are candidate tumor suppressors, because they are inactivated or down-regulated in tumors and their overexpression in a variety of cells can suppress proliferation. We evaluated the effect of *Mndal* expression in NIH3T3 cell growth in colony formation assays. This line was chosen because, as noted above, it does not contain

endogenous *Mndal*. Full-length *Mndal* was inserted into a mammalian expression vector, the resulting plasmid DNA was cotransfected with an expression plasmid containing neo resistance into NIH3T3 cells, and the G418-resistant colonies were counted 3 weeks after transfection. Consistent with a possible tumor suppressor function, the expression of *Mndal* reproducibly (8 independent experiments) decreased colony-forming efficiency by approximately 80% compared with the control vector (Figure 6). In contrast *Ifi203* decreased colony formation by approximately 40% compared with the control vector (Figure 6).

Discussion

We have identified a new interferon-inducible gene, *Mndal*, linked to Chr 1 in the same interval containing the *Pctm* modifier of PCT susceptibility, by screening for differentially expressed genes in inflammatory tissues derived from the mesenteries of TMPD-primed BALB and DBA mice. Among the differentially expressed genes, *Mndal* had the greatest difference between the 2 strains. *Mndal* shares homology with the MNDA.

MNDA is a member of the HIN-200 family of hematopoietic interferon-inducible nuclear antigens, which includes *AIM2*, *IFIX*, and *IFI16*.²²⁻²⁴ This multigene family is found on human chromosome 1q21-24²⁵ and derives its name from a unique 200-amino acid domain conserved among its members. MNDAL is similar to MNDA and AIM2 in that it contains only one 200 amino acid “a” domain, has a nuclear localization sequence, has several putative Cdk2 sites, and is found in the nucleus. MNDAL has slightly greater homology to MNDA than to AIM2. The murine counterpart of the HIN-200 family is the *Ifi200* family (members p202-p206), which reside on the distal portion of mouse Chr 1, in the same region as *Mndal*. As with other *Ifi200* members, *Mndal* expression can be induced by interferon. *Ifi205* is the only member with the same predicted structure as *Mndal*, and it shares even greater homology to *Mndal* than to human *MNDA*.

In normal tissues from mice carrying the intact gene, *Mndal* was expressed at high levels in hematopoietic tissues and in isolated B cells. Although *Mndal* is highly expressed in the tissues of TMPD-primed BALB mice, PCT cell lines do not express it, and primary plasma cell tumors in XBP1s transgenic mice also have low levels. Because the gene remains intact in the BALB-derived

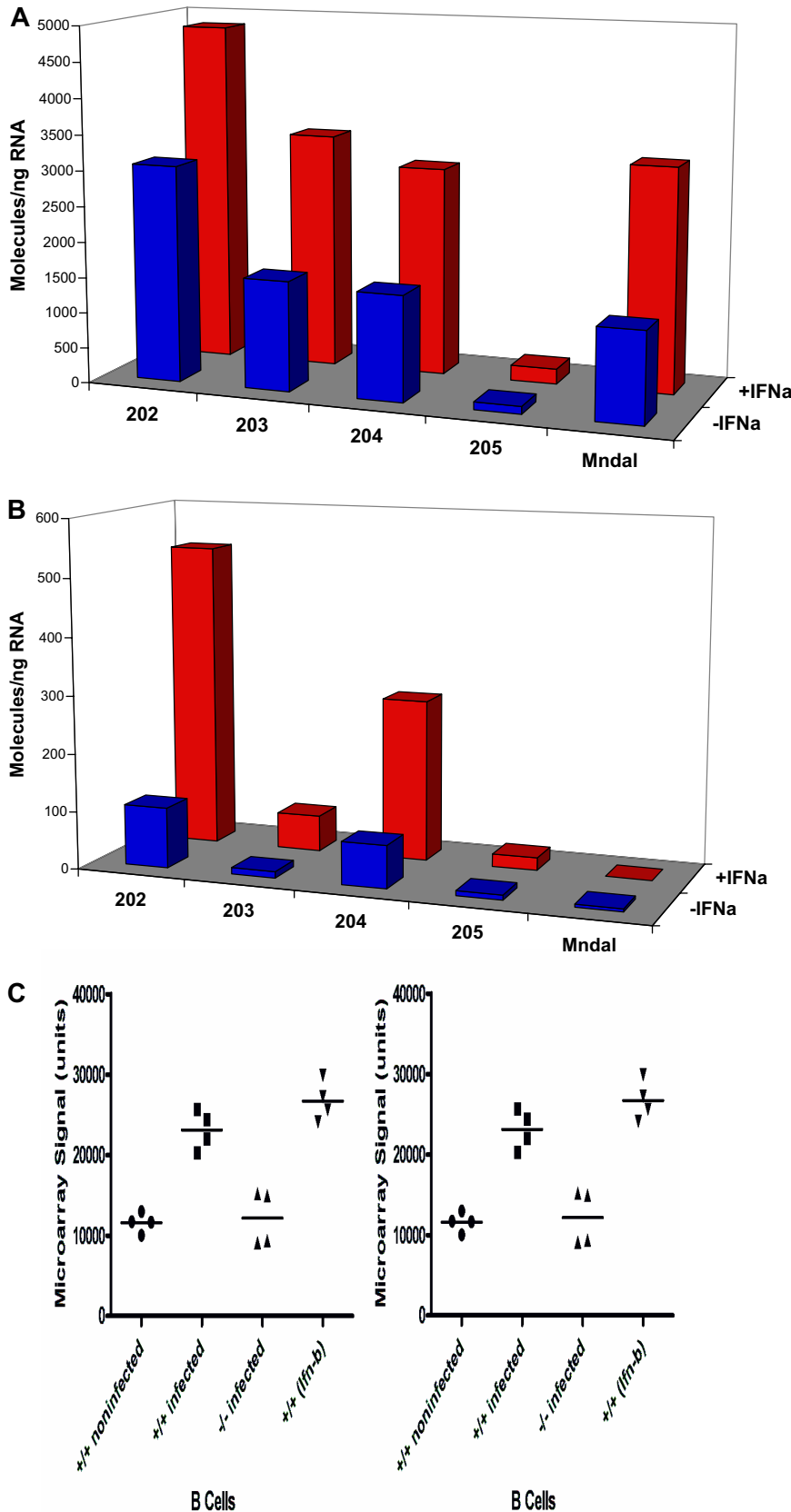


Figure 5. BALB3T3 and NIH3T3 cells were treated with interferon- α for 18 hours. Expression levels of *Ifi202*, *Ifi203*, *Ifi204*, *Ifi205*, and *Mndal* were determined by RT-PCR in the BALB3T3 (A) and NIH3T3 (B) cells. The *Mndal* gene was found to be absent by sequencing genomic DNA from NIH3T3 cells. (C) Microarray data (mined from the GEO database)²¹ collected from isolated B cells from mice that were either treated with type I interferon or infected with influenza. *Mndal* expression in B cells isolated from wild-type (+/+) mice that were either infected with influenza or treated with type I interferon- β (*Ifn*- β) were compared with control. *Mndal* expression was also examined in B cells isolated from interferon receptor knockout (-/-) mice that had also been infected with influenza.

tumor cell lines, it is possible that it has been silenced epigenetically or transcriptionally at some point during the tumorigenic process. Promoter hypermethylation of *AIM2*, another *Ifi* gene, has been found in colon cancer.¹⁵ In addition, overexpression of *Mndal*

and to a lesser extent *Ifi203* in NIH3T3 cells reduced their growth in vitro. Similar results with other *Ifi*'s, including *Ifi205*, have shown that they are also growth inhibitory and they have been proposed as tumor suppressors. *Mndal* and/or *Ifi203*, therefore,

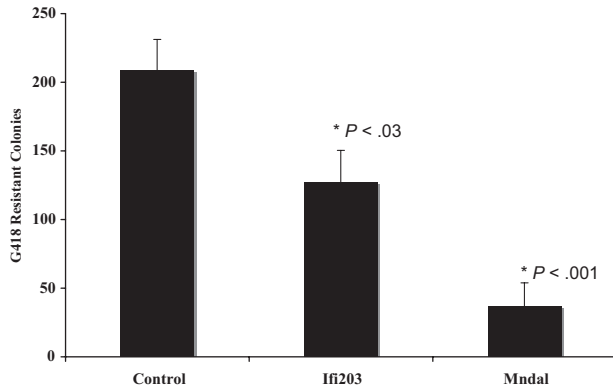


Figure 6. Colony formation in NIH3T3 cells is suppressed when transfected with *Mndal* or *Ifi203* compared with empty vector. The results of 8 separate experiments are shown. SEs are indicated, and *P* values represent *t* test comparisons with the control plasmid.

become a candidate gene(s) for the *Pctm* locus that modifies PCT susceptibility.

Further studies will be needed to confirm *Mndal*'s role in plasmacytomagenesis. However, its membership in the *Ifi200/HIN-200* family increases the plausibility of *Mndal* being a tumor suppressor gene. Overexpression of several *Ifi200/HIN-200* members has been reported to inhibit growth, by cell cycle arrest, apoptosis, or senescence, and some members have been reported to interact with *Rb* and *p53*.^{16,20,26} Members of the *HIN-200* family are somatically inactivated in several solid tumors, including breast,¹⁴ prostate,¹³ melanoma,²² and colon.¹⁵ *Mndal* is the first murine *Ifi200* gene implicated in B-cell tumor formation in vivo either as a modifier gene or possibly by some form of somatic inactivation.

Little is known about the role of the *Ifi/HIN* genes in B-cell neoplasia. Interferon- α treatment has been shown to be effective in inducing apoptosis in patients with myeloma and other B-cell malignancies.²⁷ Treatment of Daudi Burkitt lymphoma cells with interferon- α induced both *MNDA* and *IFI16*, which led to increases in p21 and *Rb* and decreases in c-Myc levels.²⁸ More recently, high expression of *MNDA* has been associated with good outcome in patients with B-cell chronic lymphocytic leukemia.²⁹

Because of the high levels of *Mndal* expression relative to the other *Ifi* family members in tissues from adult BALB mice, *Mndal* may be the predominant *Ifi200* gene in those strains that harbor an intact gene. The presence or absence of *Mndal* could have important implications for phenotypes associated with the *Ifi200* cluster, especially because *Ifi200* genes may have partially overlapping functions.

Our RT-PCR studies determined that *Ifi202* to *Ifi205* and *Mndal* expression in BALB was induced by TMPD, with a possible increase in *Ifi205* and *Mndal* relative to the other members. This observation is congruent with the finding that lymphoid tissue from TMPD-induced granulomas contains activated dendritic cells,³⁰ as well as Ly6C^{high} monocytes,³¹ both of which produce type I interferon which in turn would induce *Ifi* genes. In DBA mesenteries, both *Ifi202* and, to a lesser extent, *Ifi204/Ifi205*, levels increased by day 18; it is possible that the enhanced expression of these genes compensates for the absence of *Mndal* and low levels of *Ifi203* seen in DBA. Given the nature of the genomic segment missing in DBA and the substantially lower expression of *Ifi203* in DBA relative to BALB, it appears that the *Ifi203* promoter is partially defective in DBA mice and thus produces a compound defect affecting 2 genes simultaneously in the cluster.

Our data indicate that many inbred mouse strains resemble BALB in having an intact *Mndal* gene, whereas others resemble DBA in the absence of the gene. The absence of *Ifi* genes from the germline has not been documented before; human *AIM2* sequences have been found missing in tumors but not in the germline.¹⁵ Although we have not examined *Ifi203* in strains other than BALB and DBA, it seems likely that the *Ifi203* promoter deletion we have identified in DBA that is contiguous with the absence of *Mndal* may also be polymorphic.

The 2-megabase region of mouse Chr 1, harboring the *Ifi200* genes as well as *Spna1*, *Apcs*, *Slam*, and Fc receptor genes, has been proposed to represent a highly conserved antigen presentation cluster, reminiscent of the 6p MHC region, which may be dedicated to lipid/glycolipid antigens rather than antigen-derived peptides.³² Asefa et al²⁰ and Ludlow et al³³ have both hypothesized that this region of Chr 1 arose by gene duplication,^{20,33} and Ludlow et al³³ predicted an additional 4 genes (p207-210) in the cluster from the then current builds of the human and mouse genomes. From analyzing the predicted protein structures of IFI207 to IFI210,³³ that encoded by *Mndal* is distinct from the predicted p207 to p210 proteins.

It is tempting to speculate that *Mndal* (and/or *Ifi203*) could play a role in diseases, such as lupus, in which susceptibility loci have been mapped near this interval. The mouse *Ifi202* and human *IFI16* genes have been implicated in the development of this B cell-mediated autoimmune disease.^{12,34-37} Increased levels of *IFI16* have been reported in patients with lupus.^{36,38} In mouse models of lupus, it has been hypothesized that overexpression or high levels of *Ifi202* lead to an increase in the development of autoantibodies in response to apoptosis.^{12,39} NZB mice are susceptible to lupus, and one of the lupus susceptibility genes in NZB, designated *Nba2*, was mapped to mouse Chr1, in the same interval as the *Ifi200* gene cluster.¹² In fact, gene expression profiling studies examining a limited 11 000 genes/probe set, uncovered *Ifi202* as being overexpressed in the congenic strain carrying the NZB *Nba2* locus.^{12,40} The Affy probe set for *Mndal* was not included in this original screen. Because *Mndal* is absent in the NZB strain, examining its potential role in lupus susceptibility could be worthwhile.

In summary, *Mndal*, a new interferon inducible gene with tumor suppressor activity that is expressed at higher levels than other family members, has been found to reside in a highly polymorphic region of the genome implicated in several autoimmune and cancer phenotypes. Determining the various biologic activities and the conditions that are likely to induce these various diseases has the potential to yield new insights into their pathogenesis and treatment.

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Authorship

Contribution: K.Z. designed and performed research, collected and analyzed data, and wrote the manuscript; D.K. collected and analyzed data; W.D., R.R., W.C.V., and S.Z. performed research;

V.B. analyzed data; and B.A.M. designed research, analyzed data, and wrote manuscript.

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