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The role of Lin28b in myeloid and mast cell differentiation and mast cell malignancy

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Abstract

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Conflict-of-interest

The authors declare no competing financial interests.

Authorship

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Mast cells are critical components of the innate immune system and important for host defense, allergy, autoimmunity, tissue regeneration, and tumor progression. Dysregulated mast cell development leads to systemic mastocytosis, a clinically variable but often devastating family of hematologic disorders. Here we report that induced expression of Lin28, a heterochronic gene and pluripotency factor implicated in driving a fetal hematopoietic program, caused mast cell accumulation in adult mice in target organs such as the skin and peritoneal cavity. *In vitro* assays revealed a skewing of myeloid commitment in LIN28B-expressing hematopoietic progenitors, with increased levels of LIN28B in common myeloid and basophil-mast cell progenitors altering gene expression patterns to favor cell fate choices that enhanced mast cell specification. In addition, LIN28B-induced mast cells appeared phenotypically and functionally immature, and *in vitro* assays suggested a slowing of mast cell terminal differentiation in the context of LIN28B upregulation. Finally, interrogation of human mast cell leukemia samples revealed upregulation of LIN28B in abnormal mast cells from patients with systemic mastocytosis (SM). This work identifies Lin28 as a novel regulator of innate immune function and a new protein of interest in mast cell disease.

Introduction

Mast cells (MCs) are key effectors in allergic responses, expressing (along with basophils) the high-affinity receptor for IgE (FcεRI). Crosslinking FcεRI on tissue MCs initiates the immediate hypersensitivity reaction, with local release of histamine and inflammatory cytokines. This supports innate immune defense against infections and plays an important role in autoimmunity (1–4). Aside from their central role in allergy and inflammation, it is increasingly clear that MCs play a pivotal role in tissue regeneration and tumor remodeling (5–9).

Dysregulated MC development and activation leads to mastocytosis, a poorly-understood group of myeloproliferative neoplasms characterized by abnormal growth and activation of immature MCs and their precursors. The WHO recently classified mastocytosis into seven variants (1–4,10), ranging from cutaneous mastocytosis to mast cell leukemia (MCL). These are highly clinically variable, with median survival rates of 2 months for MCL (11,12) but virtually no mortality for mild forms. Mastocytosis is characterized by upregulated c-Kit signaling (13) and the vast majority of systemic mastocytoses harbor an imatinib-insensitive activating c-KIT mutation (usually D816V) (14–17), but this cannot explain the wide clinical variability. Understanding normal MC development and its dysregulation in SM is of central importance to developing new therapies for these disorders.

In contrast to other myeloid lineages, relatively little is known about MC development, in part because MCs are rare and difficult to isolate. Developing mast cell progenitors (MCPs) circulate through the bloodstream and only complete differentiation after migrating into skin, heart, lung, and other target organs (18–20). MCPs arise from lineage-negative (Lin^-) c-kit^+Sca-1^-myeloid progenitors (MPs) in the bone marrow, although there is controversy regarding their specific lineal relationship with other myeloid precursors (18,21,22). MC development and differentiation is influenced by the balance between core myeloid transcription factors such as C/EBPα, MITF, GATA-1, PU.1, and GATA-2, and responsive

to signals elaborated by PLA2G4 and PI-3K (19,23–26). During maturation, MCs upregulate c-kit and FcεRIαand induce expression of neutral granule components such as carboxypeptidase A3, chymase, cathepsin G, granzyme B, and the tryptases (2).

The heterochronic RNA-binding factor Lin28 is highly expressed in embryonal tissues (27–29) and, along with Oct4, Sox2, and Nanog, reprograms somatic fibroblasts into pluripotent stem cells (30). Lin28 has been heavily studied in tumorigenesis (28,29,31–34), and has been implicated in obesity (35), metabolism (36), and tissue regeneration (37). Mammals express two isoforms of Lin28 (a and b). Both proteins can enforce proliferative programs and oppose cellular differentiation, and can have similar physiological functions, although it is clear that each protein has unique properties as well (reviewed in (27)). Although the canonical downstream effect of both isoforms is to inhibit biogenesis of the *let*-7 family of microRNAs, other functions have also been ascribed to these proteins (38,39). Recent studies have begun to examine the role of LIN28B, in particular, in hematopoiesis (40–43), and indicate that reactivation of *LIN28B* in adult blood cells can revert their phenotypes to an immature stage and upregulate a fetal hematopoietic program resulting in fetal globin expression and increased production of "primitive" γδ T and B-1 B cells. A physiologic role for LIN28B in hematopoietic development remains uncertain; knockout model development is challenged by redundancies in the Lin28 isoforms and the essential role of these genes in embryonal development.

The role of Lin28 in solid tumors is well documented (28,29,32–34), but its association with hematologic malignancy is largely undefined. Some reports suggest that LIN28B overexpression can result in lymphoid malignancy (44,45), whereas other studies have not supported a role for Lin28 in hematologic malignancy (43,46). We show here that expression of LIN28B in adult mice drives accumulation of immature MCs, resulting in an overabundance of immature MCs that are hypofunctional upon antigen challenge *in vivo*. We further demonstrate that LIN28B acts at multiple stages of MC development, both to favor MC fate choice and to impair terminal differentiation. Finally, we find that LIN28B is highly overexpressed in abnormal MCs from patients with ASM; this and the proliferation of immature MCs characteristic of SM mirror our animal model findings. Together, these data implicate aberrant Lin28 expression in mast cell disease and dysfunction.

Methods

iLIN28B and ilet7 mice

iLIN28B and ilet7 mice have been described (36) and were maintained on a C57BL/6 background. *LIN28B* or *let-7* transgenes were induced with 1mg/mL of doxycycline in drinking water for 2 weeks. Unless otherwise stated, mice were 6–12 weeks old at induction. Bone marrow transplant recipients were induced for 2 weeks after 16–20 weeks of post-transplant recovery. All control mice were age- and gender-matched.

Flow cytometric analysis

Mononuclear cells harvested from hematopoietic tissues, peritoneal lavage, *in vitro* cell culture, or methylcellulose were stained with directly-conjugated antibodies or with

biotinylated antibodies followed by fluorophore-conjugated streptavidin, then analyzed by flow cytometry on a BD LSRII or BD AriaII (Becton Dickinson, East Rutherford NJ). Data were analyzed using FlowJo (Treestar Industries, Ashland OR). Antibodies are listed in Supplemental Methods.

In vitro methylcellulose cultures

FACSorted progenitors were plated in Methocult m3434 (Stem Cell Technologies, Vancouver BC) at 100–1000 cells/1.5mL/3cm dish and cultured for 10 days. Colonies were counted and morphologically categorized as CFU-G, CFU-M, CFU-GM, CFU-GEMM, or CFU-E/BFU-E before harvest for further analysis.

Bone marrow-derived mast cell cultures

Bone marrow was isolated from uninduced C57BL/6, iLIN28B, and ilet-7 mice and cultured in IMDM supplemented with 10% FCS, 50ng/mL rmSCF, and 10ng/mL rmIL-3 (R&D Systems, Minneapolis MN). Cultures were induced with 1μg/mL doxycycline. After 5 weeks, >95% of suspended cells from wild-type mice displayed MC morphology and granulation and were toluidine-blue positive and c-kit^{hi}FcεRIα^{hi} by flow cytometry.

Histologic evaluation

Cytospins were prepared using mononuclear cell suspensions derived from hematopoietic tissues or *in vitro* cell culture on a Shandon Cytospin 2 (Thermo Fisher Scientific, Waltham MA) and stained with Wright-Giemsa (Sigma-Aldrich, St. Louis MO). Peripheral blood smears were hand-drawn on glass slides and stained with Wright-Giemsa. MCs were stained with acid toluidine blue solution after fixation in Mota's solution. Tissues were fixed in neutral-buffered formalin and processed by the DF/HCC Specialized Histopathology facility.

Electron Microscopy (*EM*)

Cell pellets were fixed in 2.5% glutaraldehyde, 1.25% paraformaldehyde, and 0.03% picric acid in 0.1M sodium cacodylate (pH 7.4). Subsequent preparation and EM were performed at the EM Core Facility at Harvard Medical School.

Bone marrow transplantation

Mononuclear cells were harvested from the bone marrow of age-and-gender matched male or female mice and either directly retroorbitally injected(1×10^6 cells/recipient) or stained using SLAM markers, FACSorted for LT-HSCs (Lin$^-$Sca-1$^+$c-Kit$^+$CD150$^+$CD48$^-$), and then injected into anesthetized, lethally-irradiated (950 rads) congenic (CD45.1$^+$)recipients, either with competitorCD45.1$^+$BM cells or with 3×10^5CD45.1$^+$helper cells to avoid radiation-induced hematologic failure.

Passive cutaneous anaphylaxis (PCA) assay

PCA assays were performed according to standard protocols (47). Briefly, control and iLIN28B mice were induced for 2 weeks and the near pinnae were injected with PBS (control) or anti-dinitrophenol (DNP) IgE. 24h later, mice were tail-vein injected with DNP-

human serum albumin (HSA) in a solution of Evans blue dye; histamine release resulted in increased vascular permeability and dye extravasation into the ear pinna. Pinnae were removed, macerated, and incubated overnight in acetone or formamide. After incubation, 570nm absorbance of the supernatant was spectrophotometrically measured. Positive control pinnae were injected directly with histamine followed by DNP-HSA in Evans blue dye.

RT-PCR

RNA extraction from Trizol and RT-PCR were carried out according to manufacturer's instructions (Life Technologies, Carlsbad CA). Banked patient samples were obtained through Dana-Farber Cancer Institute (DFCI) IRB protocol 01–206 (R. Soiffer, PI). Bone marrow aspirates were centrifuged for buffy coat isolation, then subjected to dextran erythrocyte sedimentation followed by lysis in ammonium-chloride-potassium buffer. Subsequently, bone marrow mononuclear cells were stained with antibodies to c-Kit, CD25, CD2, and CD45; abnormal MCs (as defined in the patients' medical records) and control cells were FACSorted into Trizol for further analysis. Primer sequences are provided in Supplemental Methods.

Results

Lin28b is normally downregulated upon mast cell differentiation in adult mice

Recent analyses have implicated Lin28b in the specification of primitive hematopoietic lineages during fetal development, and shown that it is highly expressed in mouse fetal liver but that expression declines during postnatal life ((42); https://gexc.stanford.edu/search). To evaluate the possible involvement of Lin28b in post-natal mast cell development, we first examined Lin28b expression during MC differentiation in bone marrow-derived mast cell cultures (BMMCs). MC development is poorly understood, and its kinetics are difficult to study *in vivo*; the BMMC system represents a very useful and well-accepted model system for the analysis of MC development and function (48).In addition, BMMCs permit the synchronous generation of large numbers of MCs, enabling kinetic evaluation of MC development. Lin28b transcription decreased over time in BMMC culture (Fig 1), indicating that changes in Lin28 levels may be important for MC maturation.

Expression of LIN28 Bleads to increased numbers of mast cells and their progenitors

To study further the role of Lin28b in MC development, we used inducible transgenic mice that express LIN28B upon doxycycline treatment (iLIN28B mice (36)). After a two-week induction (Fig. 2A), hematopoietic cells were harvested from spleen, bone marrow, peritoneal cavity, and peripheral blood and analyzed by histology and flow cytometry. LIN28B-expressing mice harbored greater numbers of peritoneal MCs, identified as c-kit^{+}FcεRIα^{+},than age- and gender-matched controls (5.22±0.287% of live peritoneal cavity cells in iLIN28B mice (n=12) vs. 2.98±0.202% in control C57BL/6 mice (n=7), Fig 2B). Sectioning and staining of ear pinnae revealed a similar (~1.5-fold) increase in MCs in the skin of induced iLIN28B mice as assessed by toluidine blue staining (Fig 2C, arrowheads). iLIN28B mice also had a 3.6-fold increase in splenic mast cell precursors (MCPs)(Fig 2D, 0.055±0.006%of live cells in iLIN28B (n=12) vs. 0.015±0.002% in controls (n=7)) and twice as many bone marrow MCPs (Fig 2E, 0.122±0.009% of live cells in iLIN28B mice vs.

0.064±0.005% in controls, n=9 of each genotype) as controls. MCPs were defined as lin$^-$c-Kit$^+$F$_c\gamma$RII/III$^+$int$\beta7^+$ in the spleen and lin$^-$Sca −1$^-$c-Kit$^+$CD150$^-$ Flk2$^-$ int$\beta7^+$CD27^{dim} in the bone marrow (18,20).

Lin28 expression promotes mast cell progenitor accumulation

To understand how Lin28 expression augments MC numbers, we next examined MC developmental stages in iLIN28B mice. MC provenance is not completely understood, and MCs appear capable of developing from several different progenitors (18–21,49). However, the most exhaustive studies indicate that MCs develop predominantly from the megakaryocyte-erythroid progenitor (MEP) pool of myeloid progenitors (18,20). Interestingly, induction of LIN28B caused skewing of the myeloid progenitor compartment in the marrow of iLIN28B mice, with significantly higher percentages of MEPs than in controls (Fig 3). This increase in MEPs was accompanied by decreases in the proportions of myeloid progenitors (MPs) that were granulocyte-macrophage precursors (GMPs, Fig 3), although the percentage of live cells that were GMPs was less affected. There were no statistically significant differences in the proportions of cells in the common myeloid progenitor (CMP) pool, suggesting that Lin28 may act at the level of CMP fate choice. In keeping with these findings, mice induced to express *let-7* for two weeks were found to have fewer numbers of bone marrow MCPs compared to controls (Fig S1, 0.01±0.003% vs. 0.048±0.007%, p=0.007). Because the lifespan of fully differentiated MCs exceeds the 2 week induction period used (50,51), we did not expect to see differences in the frequencies of peritoneal MCs. However, a trend towards reduced peritoneal MC numbers was observed (1.88±0.3% vs. 2.39±0.02% of live cells; p=0.16) (Fig S1). Taken together, these data indicate that Lin28b expression acts to promote MC lineage commitment at the CMP→MEP transition.

The mast cell phenotype in iLIN28B mice is intrinsic to hematopoietic cells

To determine whether the effects of LIN28B expression on MC development in iLIN28B mice were driven by LIN28B expression in MC precursors themselves, we sorted Lin$^-$Sca-1$^+$c-kit$^+$(LSK) progenitor cells from induced iLIN28B bone marrow and cultured them in methylcellulose media containing doxycycline and cytokines that support unbiased myeloid differentiation (m3434, Stem Cell Technologies, Vancouver BC). After 10 days, LSK cells from iLIN28B mice that differentiated in culture generated predominantly c-Kit$^+$FcεRI^{lo}MC progenitors (Fig S2), whereas control LSKs produced a variety of different myeloid cell types. This result indicates that the MC-promoting effects ofLIN28B induction *in vivo* are maintained *ex vivo* in differentiating hematopoietic progenitors and suggests that LIN28B induction may delay or impair MC terminal differentiation in a cell autonomous manner. However, because the cells assayed in these cultures were harvested from mice in which LIN28B had already been induced *in vivo*, it remained possible that durable cell-extrinsic effects of prior induction were carried forward into the *in vitro* setting. Thus, we performed a further, definitive *in vivo* experiment by analyzing iLIN28B hematopoietic chimeras generated by transplantation of bone marrow from uninduced LIN28B-transgenic mice into lethally-irradiated CD45.1 congenic recipients. After full hematopoietic reconstitution (16–24 weeks), recipient mice were induced to express LIN28B for 2 weeks and analyzed for MC phenotypes. We again found increased numbers of MCPs in the spleen

(0.05±0.005% of live splenocytes in iLIN28B chimeras vs. 0.03±0.005% in controls, n= 10 in each group) and bone marrow of iLIN28B chimeric mice (0.15±0.008% of live marrow mononuclear cells in iLIN28B chimeras vs. 0.07±0.007% in controls, n=10 in each group; Fig 4, panels B and C). These results mirror those obtained in induced, transgenici LIN28B mice (Fig 2, panels D and E).

MCs are long-lived (50,51), and recipient peritoneal MCs persisted *in situ* in hematopoietic chimeras, preventing donor reconstitution in the peritoneum. Therefore, another transplantation experiment was performed using recipients that were injected intraperitoneally with distilled water at the time of LIN28B induction to lyse resident recipient MCs (50). Increased numbers of donor MCs were found in the peritoneal lavage of recipients receiving iLIN28B bone marrow as compared to control marrow (4.94±0.66% of live peritoneal mononuclear cells in iLIN28B chimeras vs. 2.49±0.48% in controls, n=5 for each group, Fig 4D). These findings, taken together, indicate that the effects of LIN28B in promoting MC fate choice are intrinsic to the hematopoietic compartment.

Expression of LIN28B impedes terminal mast cell differentiation

To investigate further the finding that LIN28B-induced LSK cells are impaired in their ability to differentiate fully into MCs in methylcellulose culture, we studied the kinetics of c-kit and FcεRIαreceptor expression, as well as transcriptional and ultrastructural features of developing MCs in iLIN28B and control BMMC cultures. These experiments corroborated our methylcellulose culture studies and demonstrated that, *in vitro*, LIN28B-expressing BMMCs matured with delayed kinetics relative to control BMMCs (Fig 5A). Concordantly, acquisition of toluidine blue staining in cultured cells (Fig 5B) was delayed. Furthermore, electron microscopy demonstrated a decrease in the number of mature, electron-dense granules in LIN28B-expressing BMMCs (Fig 5C and D, 7.8±1.06 v. 16.83±1.62 per cell at d32 of culture, n=25 cells, representative of BMMCs from 3 different mice per condition), reminiscent of features reported in fetal liver-derived MCs and in human MC disease (52,53).Thus, these data suggest that induced LIN28B expression prevents MCs from fully maturing.

iLIN28B mast cells are hypofunctional

Delayed kinetics of LIN28B-expressing mast cell development were not apparent *in vivo*, although increased numbers of mast cell progenitors are consistent with slowed maturation. *In vivo* development is likely too asynchronous to permit kinetic analyses, particularly as *in vitro* iLIN28B BMMCs ultimately attained the same immunophenotypic endpoint as controls (Fig 5A). To evaluate mast cell differentiation *in vivo*, we hypothesized that differences in developmental kinetics might have functional manifestations. To test this, we examined MC histamine release in end organs by performing passive cutaneous anaphylaxis (PCA) assays. Recipient tissue MCs persist through bone marrow transplant, precluding performance of these assays in transplant recipients. Therefore, we analyzed MC function in iLIN28B transgenic mice. The histamine response to a mast cell-specific IgE-mediated stimulus was attenuated in iLIN28B mice relative to controls (Fig 6A). Histamine treatment *per se* caused equivalent increases in vascular permeability in control and iLIN28B mice (Fig S3), indicating that the diminished extravasation seen in response to MC-specific

stimuli was not attributable to an inability of iLIN28B vasculature to respond to histamine. Thus, LIN28B-expressing MCs exhibit an impaired response to IgE signaling *in vivo*, consistent with a failure to mature fully. To test this further, we interrogated MC cytokine responses to IgE-mediated receptor stimulation in BMMC cultures from control and iLIN28B mice that were induced to express LIN28B *in vitro* at the initiation of the cultures, but not before. iLIN28B BMMCs produced less TNFα and IL-6 in response to FcεRIα stimulation than controls (Fig 6B), confirming that iLIN28B MCs are intrinsically hypofunctional.

LIN28B downregulates *C/ebpa* expression

To investigate the transcriptional program underlying the production of immature MCs in LIN28B-expressing mast cell precursors, we performed RT-PCR analysis of RNA isolated from BMMCs of induced or uninduced iLIN28B mice to evaluate genes important in MC development and function. We confirmed that iLIN28B BMMCs expressed high levels of LIN28B and low levels of *let-7* species (Fig 7A), consistent with increased LIN28B activity. Transcription of the *let-7* target *Hmga2* was not significantly elevated (Fig 7B), which may explain why induced iLIN28B animals do not develop mast cell malignancies (see below). The *let-7* target *Igfbp2* was not expressed in BMMCs, with or without LIN28B induction (not shown). However, iLIN28B BMMCs expressed less *Fcer1a* as determined by flow cytometry (Fig 7B). Additionally, although expression of MC genes such as *Kit* and *Gata-1* was not substantially altered, LIN28B-expressing BMMC cultures had significantly decreased expression of *C/ebpa* (0.23±0.07 relative to controls; Fig 7B). C/ebpα is a key regulator of granulocyte differentiation (54,55), and especially of basophil differentiation (25). Previous studies have demonstrated that fate specification at the level of the basophil-MCP is dictated by the balance between C/ebpα and MITF, with C/ebpαspecifying basophil fate(25). Thus, downregulation of C/ebpαin iLIN28B BMMCs would be expected to favor MC fate choice. Expression of *Mitf* was also decreased (0.63±0.07 fold relative to controls; Fig 7B), although to a lesser extent than the decrease in *C/ebpa*. Thus, expression of LIN28B results in downregulation of genes critical for terminal differentiation of basophils and mast cells, with preferential downregulation of the basophil-specification branch of that program. These molecular alterations likely underlie the accumulation of immature mast cells seen in iLIN28B mice.

We also performed transcriptional analysis on myeloid progenitors from iLIN28B mice. Adult myeloid progenitors represent an earlier stage of development than the cultured BMMCs, and do not express Lin28b at appreciable levels (not shown, but concordant with published datasets at https://gexc.stanford.edu/search and http://www.immgen.org/databrowser/index.html). Enforced expression of LIN28B in CMPs resulted intranscriptional changes distinct from those seen in BMMCs. Although *Fcer1a* expression was low in iLIN28B CMPs just as in iLIN28B BMMCs, *C/ebpa* levels in CMPs were comparable to controls. Furthermore, *Gata-1* expression was higher in iLIN28B CMPs than in controls (Fig 7C). Gata-1, a master regulator of erythropoiesis, is also critical for differentiation of the megakaryocyte, eosinophil, and mast cell lineages (24), and ectopic expression of Gata-1 converts lymphoid and myelomonocytic precursors into megakaryocyte/erythroid precursors (56).These findings indicate that induced LIN28B in mast cell progenitors augments mast

cell production through distinct mechanisms acting at different stages of development:first, it promotes CMP differentiation to form MEP over GMP by upregulating *Gata-1*, and second, it slows terminal differentiation while favoring MC fate choice at the basophil-MCP level by downregulating *C/ebpa* more than *Mitf*.

Human systemic mastocytosis overexpresses LIN28

Lin28 has been implicated in tumorigenesis (28,29,31–33), and the accumulation of immature MCs in animals induced to express LIN28B suggested a potential role for this protein in MC disease. We therefore analyzed dysplastic MCs from patients with SM/MCL treated at the DFCI. SM is rare, and patients often have a low disease burden in comparison to other hematologic malignancies (10,15). Additionally, dysplastic MCs are difficult to dislodge from the bone marrow, such that aspirates frequently contain very few (<10%) abnormal MCs (57). Between 2010 and 2014, 19 patients with SM/MCL were enrolled in the DFCI hematologic malignancy registry. We were able to obtain primary samples from four of these patients, two of whom were treated with midostaurin, a tyrosine kinase inhibitor with multiple targets including c-Kit (Table I)(58,59). Bone marrow mononuclear cells from these patients were stained with antibodies for abnormal mast cell markers previously identified by clinical flow cytometry, including c-Kit, CD45, CD25, and CD2. Cells were sorted and analyzed by RT-PCR for *LIN28B* expression. Interestingly, and in contrast to the iLIN28B animal model, *LIN28B* and *HMGA2* were both found to be highly expressed (Fig. 7D) in abnormal human MCs as compared to their corresponding nondysplastic bone marrow cells, regardless of clinical subtype or c-Kit mutational status. These results implicate LIN28 as an important cofactor in the pathogenesis of human SM/MCL.

Discussion

Growing evidence highlights the central role of mast cells in innate immunity. MCs mediate inflammatory responses in multiple contexts and also coordinate the responses of other immune cells (1–3). In rare instances, MC development is subverted to cause mastocytosis or MCL, usually as a result of a somatically-acquired c-Kit mutation(14,58).However, a subset of pediatric and aggressive MC dyscrasias do not have c-Kit mutations, and MCL often loses c-Kit mutations(15,60). Here, we ascribe an important function to the RNA binding protein Lin28 in MC development and associate upregulation of this protein with aggressive mast cell malignancy.

Lin28 has been extensively studied as a pluripotency and proliferation factor that impairs cellular differentiation(28,30). We demonstrate here that enforced expression of LIN28B causes accumulation of MCPs in bone marrow as well as increased MC numbers in end organs. However, the MCs found in iLIN28 tissues are immature in both immunophenotype ($Fc\varepsilon RI\alpha^{lo}$, c-$Kit^{hi}$) and function. Our *in vitro* studies confirm that Lin28 is normally downregulated during MC development, and reveal that LIN28B induction impedes MC development and results in diminished responses to prototypical MC stimuli. iLIN28B mice also exhibit alterations in cell fate decisions at the CMP and basophil-MCP stage, associated with deregulated expression of transcription factors involved in MC specification and

differentiation. It is likely that the accumulation of MCPs *in vivo* in iLIN28B mice reflects both a slowing interminal differentiation of these cells and a skewing of cell fate decisions by progenitor cells in the myeloid lineage. It is also possible that LIN28B expression affects mast cell progenitor compartmentalization or recruitment and/or mature mast cell proliferation, and that these mechanisms augment the developmental effects of LIN28B to cause accumulation of MCPs *in vivo.*

Lin28 activity impacts a number of biological processes. The best-characterized of these is its inhibition of the biogenesis of the microRNA *let-7*, although *let-7* independent functions of Lin28 have been described(38,39). In MC development, enforced expression of *let-7* yields an opposite phenotype to that of Lin28 expression, suggesting that the effects of LIN28B in this system may be mediated in part through *let-7* (Fig. S2). However, although expression of LIN28B results in marked suppression of *let-7* (Fig 7A), canonical downstream targets such as *Hmga2* are not uniformly upregulated. This finding suggests a context-dependency to Lin28b expression, as well as *let-7* independent mechanisms of Lin28B action.

Our investigation into the role of LIN28B in MC development agrees with previous work implicating Lin28 as a factor that favors more primitive cellular phenotypes. The phenotype of MCs in adult mice induced to express LIN28B resembles the published phenotype of fetal liver-derived MCs (52). In our analyses, hematopoiesis in LIN28B-induced fetal liver showed the same bias toward MEPs at the expense of GMPs (data not shown), indicating that upregulation of LIN28B impacts myelopoiesis even when Lin28b expression is already high.

Previous studies have reported differences in the ability of ectopic Lin28 to induce hematologic malignancy(43,44,46). In our system, we did not observe malignancy in transplant recipients of mouse iLIN28B bone marrow, even after months ofLIN28B induction. Our finding that *Hmga2* expression was not increased in mouse BMMCs induced to express LIN28B may explain these discrepancies, suggesting that mechanisms downstream of *let*-7 can abrogate the oncogenic effects of Lin28b on Hmga2 in some settings. Nonetheless, LIN28B upregulation has been described in human hematologic malignancies such as blast crisis CML(29), and we describe here the marked upregulation of both LIN28B and HMGA2 in abnormal MCs from patients with SM and MCL. It is interesting to note that this upregulation occurred regardless of clinical subtype or of c-kit mutation status, suggesting that LIN28B may complement c-kit in the pathogenesis of mast cell disease. The induction of LIN28B expression in SM is intriguing and warrants further investigation, ideally on untreated primary patient samples, to determine the role of LIN28 in mast cell disorders and its interaction with the c-Kit mutation. Thus, our findings implicating Lin28b as a novel regulator of mast cell fate and function highlight the need for more extensive study of this protein in human MC disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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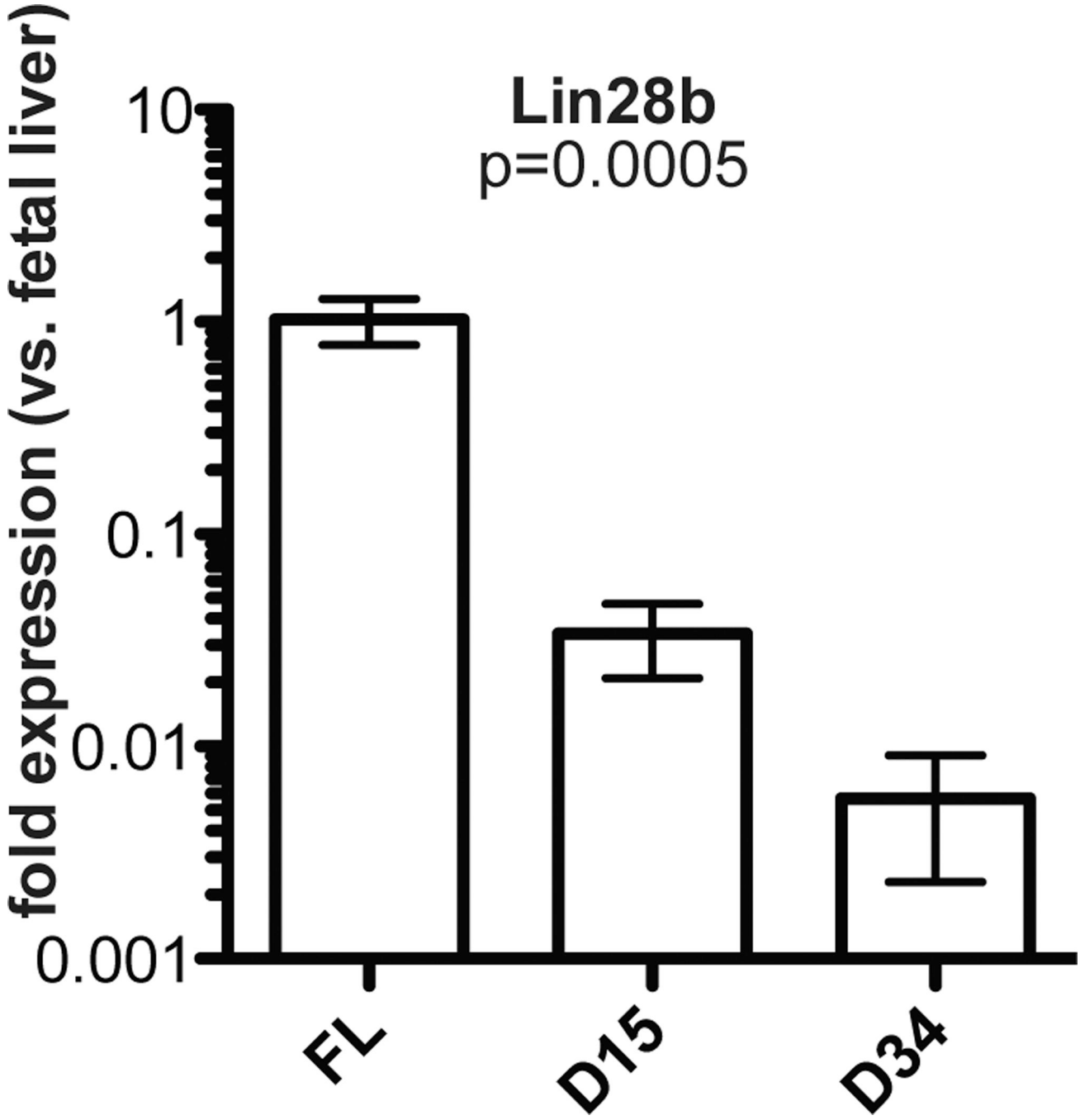

Figure 1. Lin28 plays a role in mast cell development

Bone marrow mast cell (BMMC) cultures are a well-accepted model system of mast cell development. Examination of the expression of Lin28b in BMMC cultures showed that Lin28b is downregulated as mast cells mature. Values are expressed as fold-change over expression in fetal liver, which has been shown to express high levels of Lin28b. p=0.0005 by one-way ANOVA in both cases.

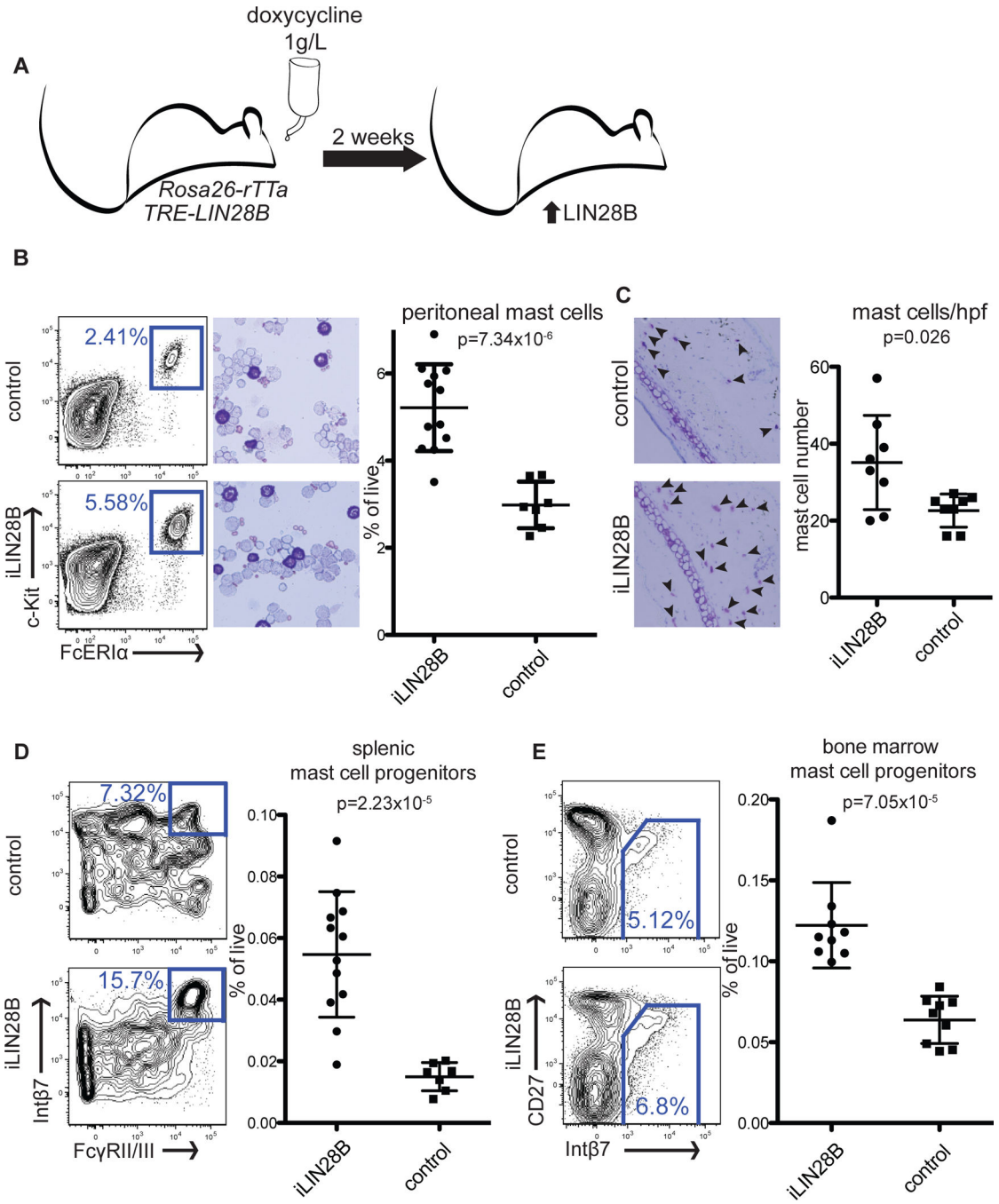

Figure 2. Induced expression of LIN28B leads to the accumulation of mast cells and mast cell progenitors

(A) iLIN28B mice, which express LIN28B under the control of a tet-on promoter, were induced with doxycycline-containing drinking water for 2 weeks and then sacrificed. (B) Induction of LIN28B leads to a 1.75-fold increase in peritoneal mast cells. Peritoneal lavage mononuclear cells were stained with antibodies to c-Kit and FcεRIα and analyzed by flow cytometry (left) or cytospun and stained with toluidine blue (right). Images were obtained on an Olympus BX60 light microscope equipped with an Olympus DP70 camera using Olympus DP Controller software; original magnification x600 for all images. (C) iLIN28B

mice have higher numbers of skin-resident mast cells than controls after induction. Ear sections were obtained and stained with toluidine blue (left), and toluidine blue (+) mast cells per high-powered field (hpf) were enumerated (right). Images were obtained on an Olympus BX60 light microscope equipped with an Olympus DP70 camera using Olympus DP Controller software; original magnification x600 for all images. (D,E) LIN28B expression results in increases in splenic (D) and bone marrow (E) mast cell progenitors (MCPs). Spleens from induced iLIN28B (circles) and control mice (squares) were harvested, processed, and stained for lineage markers, c-Kit, integrinβ7, and $F_c\gamma$RII/III; splenic mast cell precursors are lin^-c-$Kit^+F_c\gamma RII/III^+int\beta7^+$. Bone marrow mononuclear cells were stained for lineage markers, Sca-1, c-Kit, CD150, Flk2, integrinβ7, and CD27. BMMCPs are lin^-Sca-1^-c-$Kit^+CD150^-Flk2^-int\beta7^+CD27^+$.

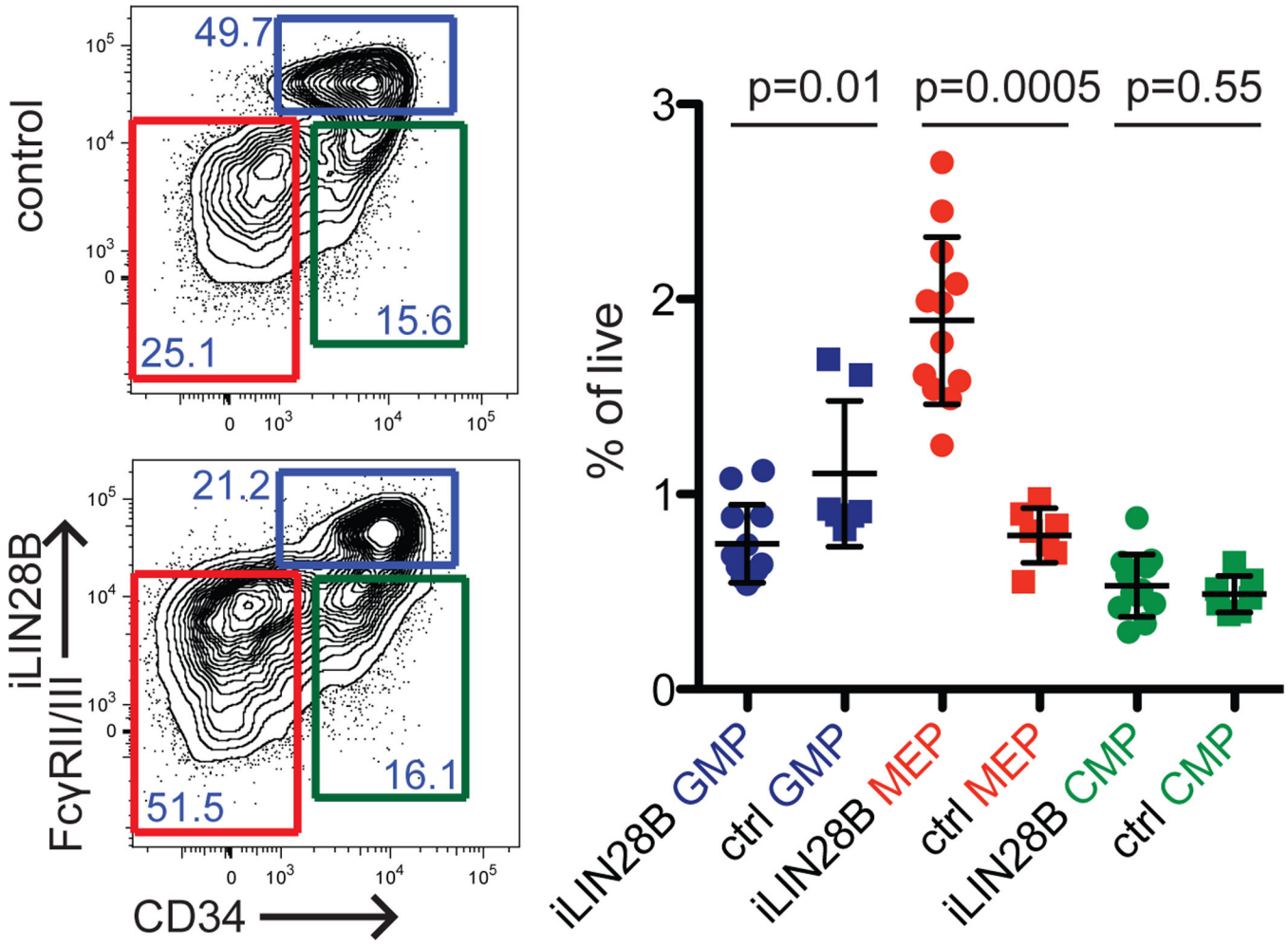

Figure 3. LIN28B induction favors the differentiation of CMPs into MEPs rather than GMPs

Bone marrow from induced iLIN28B mice or control animals was harvested and mononuclear cells were stained for lineage markers, c-Kit, Sca-1, $F_c\gamma$RII/III, and CD34. Within the $lin^-c\text{-}Kit^+Sca\text{-}1^-$ myeloid progenitor (MP) pool, common myeloid progenitors (CMPs) are $F_c\gamma RII/III^-CD34^+$ (green box) and differentiate into $F_c\gamma RII/III^+CD34^+$ granulocyte-monocyte precursors (GMPs, blue box) and $F_c\gamma RII/III^-CD34^-$ megakaryocyte-erythrocyte precursors (MEPs, red box). Overexpression of LIN28B resulted in increased percentages of MEPs and decreased percentages of GMPs (expressed as a percentage of myeloid progenitors on the left and as a percentage of all live bone marrow mononuclear cells on the right). Total numbers of bone marrow cells were not significantly different.

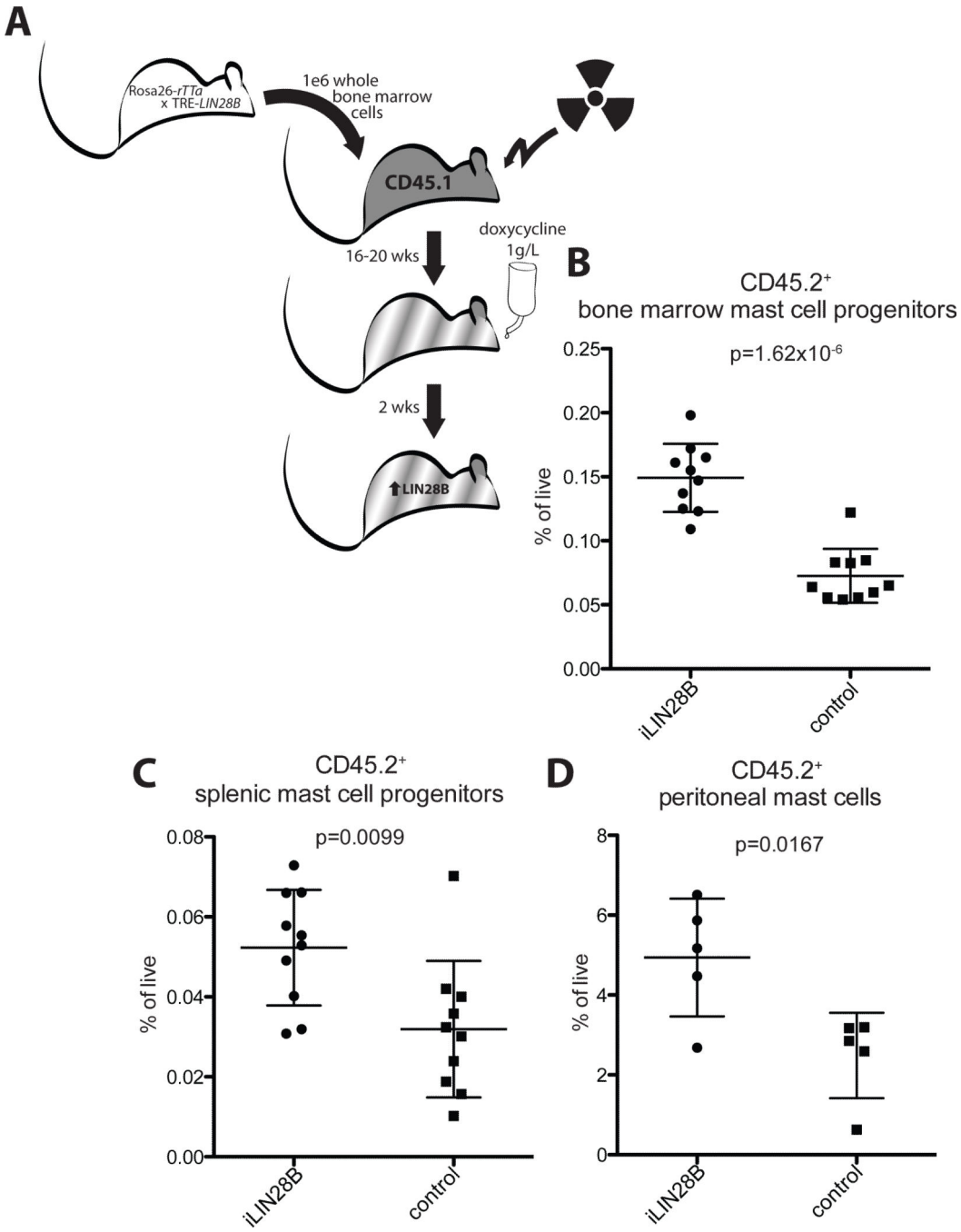

Figure 4. The effects of LIN28B overexpression on mast cell development are cell-intrinsic

(A) LT-HSCs ($Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}$) were sorted from uninduced iLIN28B (circles) and control mice (squares) and transplanted into lethally-irradiated congenic (CD45.1) recipients. After long-term hematopoietic reconstitution, recipients were induced for 2 weeks with 1g/L doxycycline drinking water and then sacrificed. (B,C) Restriction of LIN28B overexpression to the hematopoietic compartment results in an accumulation of bone marrow and splenic mast cell progenitors very similar to that seen in induced iLIN28B mice. As in Figure 2, splenic mast cell precursors are $lin^{-}c\text{-}Kit^{+}F_{c}\gamma RII/III^{+}int\beta7^{+}$. BMMCPs

are $lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}CD150^{-}Flk2^{-}int\beta7^{+}CD27^{+}$. Because peritoneal mast cells are long-lived, the majority of MC recovered from the peritoneum were of recipient origin (i.e. $CD45.1^{+}$). Thus, the transplant experiment was repeated (D) and distilled water was intraperitoneally injected at the time of induction to lyse resident recipient mast cells. After 2 weeks of induction, iLIN28B overexpression resulted in an increase in peritoneal mast percentages.

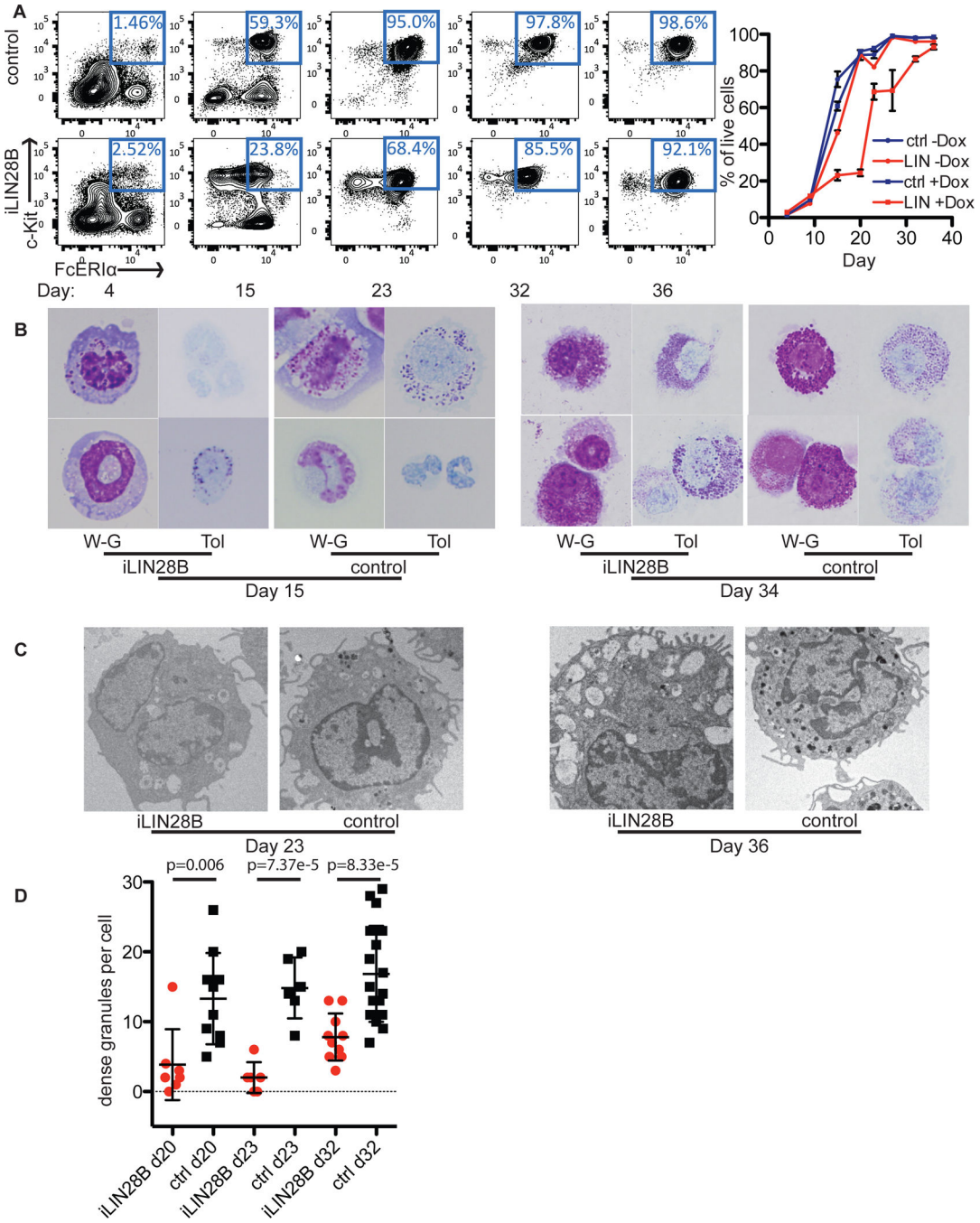

Figure 5. iLIN28B induction results in delayed maturation of mast cells *in vitro*

(A) Induction of LIN28B slows BMMC differentiation and results in an accumulation of immature c-Kit^{+}FcεRIα^{-} cells. Cultures were initiated from uninduced control or iLIN28B mice, as described, with or without subsequent induction, and samples were taken at defined time points for flow cytometric analysis. Plots represent a time course of maturation; each condition was performed in biological triplicate. These data are summarized in graphical format, showing a clear shift to the right of the immunophenotypic maturation curve of induced iLIN28B BMMC (p=0.005 by one-way ANOVA). (B) LIN28B overexpression

slows acquisition of toluidine blue-positive mast cell granules. Cells were taken from BMMC cultures at the indicated time points and cytospun, then stained with Wright-Giemsa preparation (left) or toluidine blue (right). Although iLIN28B cultures did acquire toluidine blue positivity (far right), it occurred with kinetics similar to those seen in (A). Images were obtained on an Olympus BX60 light microscope equipped with an Olympus DP70 camera using Olympus DP Controller software; original magnification x600 for all images. (C) Electron microscopy demonstrates that iLIN28B BMMCs have fewer dense granules than controls. BMMCs at the specified time points were visualized by transmission EM and their electron-dense granules enumerated by visual inspection. Differences in total granule number did not reach statistical significance whereas differences in dense granule number were highly statistically significant (D), whether expressed as number of dense granules per cell or percentage of granules that were dense per cell (not shown). Images were acquired on a Philips/FEI Tecnai 12 Biotwin/Spirit TEM equipped with an AMT XR60 camera; original magnification ×2000 for all images.

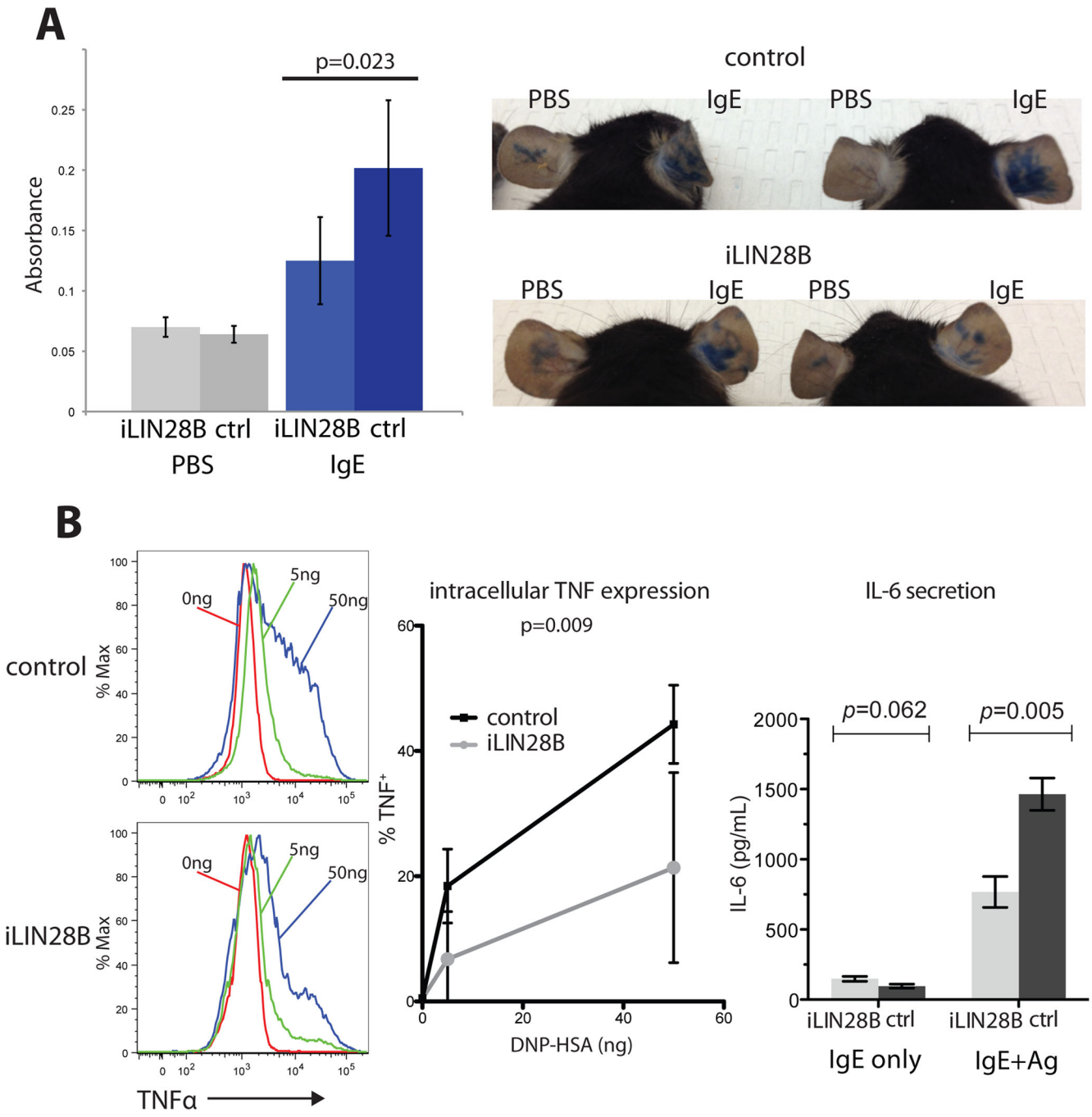

Figure 6. Mast cells from iLIN28B mice are hypofunctional

(A) iLIN28B mice have impaired IgE-mediated histamine responses. Control and iLIN28B mice were induced for two weeks with doxycycline. Their pinnae were then injected subcutaneously with PBS or anti-DNP IgE. 24 hours later, mice were intravenously injected with DNP-HSA in a solution of Evans' blue dye; local histamine release resulted in increased vascular permeability and extravasation of the dye into the ear pinna. iLIN28B mice were observed to have decreased extravasation (right panel, bottom) as compared to control mice (right panel, top) indicating that they released less histamine in response to IgE

signaling. Pinnae were subsequently removed and macerated to release the dye, and absorbance was quantitated (left panel). (B) iLIN28B mast cells have impaired cytokine production in response to IgE stimulation. Bone marrow mast cell cultures from iLIN28B and control mice were challenged with anti-DNP IgE and DNP-HSA at varying concentrations. Intracellular TNFα expression was measured by flow cytomery (left panels) and IL-6 secretion was evaluated by serum ELISA (right panel).

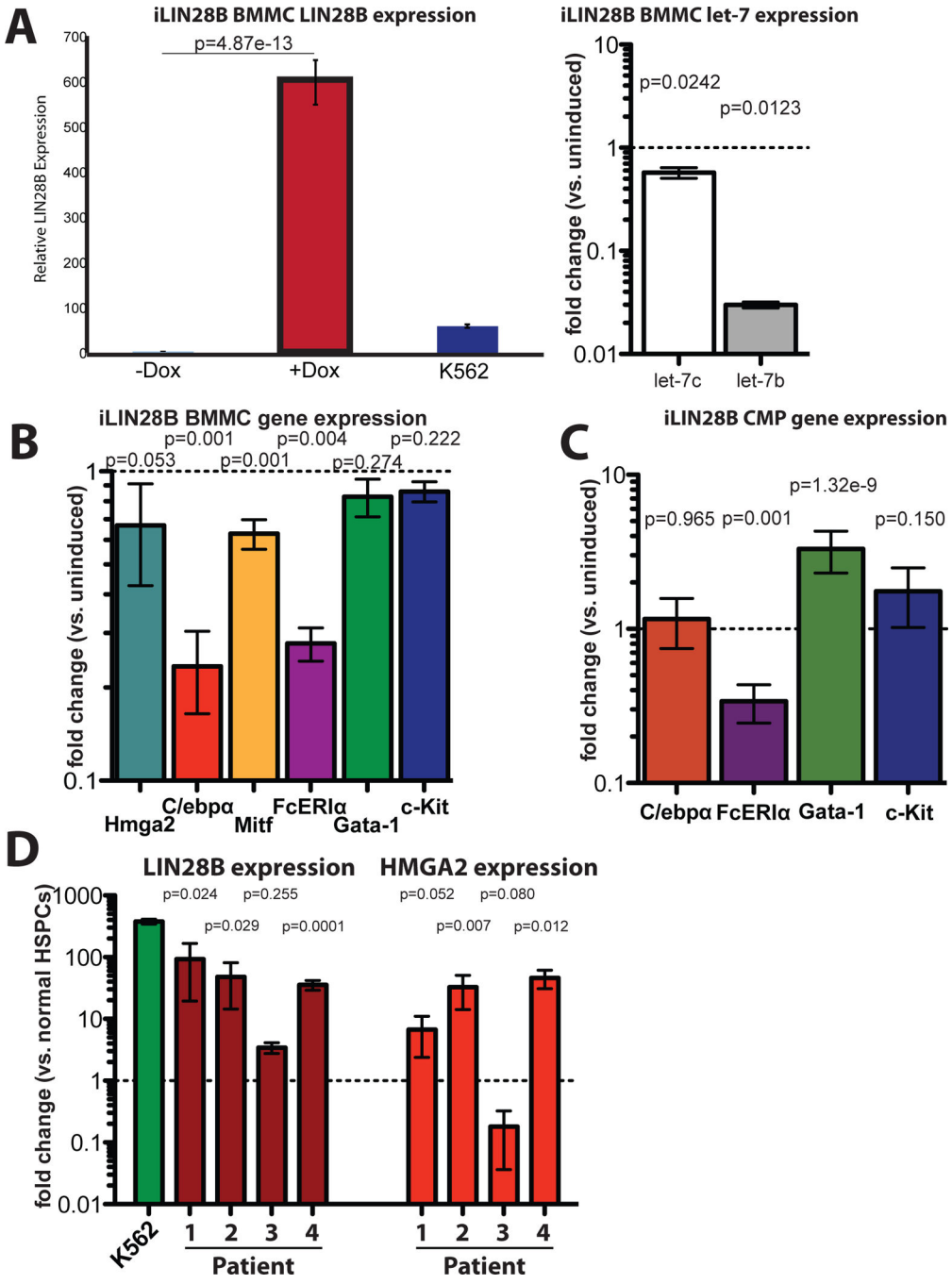

Figure 7. Induction of LIN28B favors mast cell fate choice but enforces an immature mast cell phenotype, and LIN28 is upregulated in SM and MCL

(A) (Left) RNA was extracted from uninduced (left column) or induced (center column) iLIN28B BMMCs and qPCR was performed on *LIN28B*. As expected, induction of iLIN28B BMMCs resulted in significant expression of *LIN28B*. Values are expressed as fold-change over control BMMC expression. K562 cells were used as a control (right column); fold-change is displayed on a linear y-axis. (Right) qPCR was performed for *let-7c* (left) and *let-7b* (right), and markedly decreased expression of these species was observed. Values are expressed as fold change over expression in control cultures and are displayed on

a logarithmic y-axis. (B) iLIN28B BMMCs downregulate *C/ebpa* and *Mitf* expression, indicating that Lin28 plays a role in basophil-mast cell fate choice. However, *C/ebpa* expression is significantly more downregulated than *Mitf*, such that the balance of these transcription factors skews towards the mast cell fate. Expression of mast cell genes *Gata-1* and *Kit* is not significantly different (p=0.274 and 0.222 respectively).Expression of *let*-7 target *Hmga2* is not significantly upregulated, indicating that *let-7* downregulation is insufficient for *Hmga2* upregulation. Values are normalized to control BMMC expression levels. Results are compiled from 2 separate experiments, each of which comprised 3–4 technical replicates of 3 biological replicates for each condition (n=6 mice total for each condition). Data are expressed as mean of means ±SEM, and displayed on a logarithmic y-axis. (C) As in BMMCs, CMPs from induced iLIN28B mice downregulate *Fcer1a* expression. Additionally, they upregulate *Gata-1* expression, suggesting a mechanism whereby CMPs favor an MEP over GMP fate choice. RNA was isolated from flow sorted CMPs (lin^{-}c-$Kit^{+}$$Sca\text{-}1^{-}$$Fc\gamma RII/III^{-}CD34^{+}$) from induced iLIN28B mice. Each column represents the aggregate of 4 technical replicates each from 3 biological replicates, and data are represented in terms of fold change over CMPs from control mice (±SD). Values are displayed on a logarithmic y-axis.(D) Abnormal mast cells from patients with ASM express high levels of *LIN28B* and *HMGA2*. Abnormal (+) and control (−) cells were sorted from bone marrow aspirates from patients with ASM followed at the Dana-Farber Cancer Institute according to clinically-reported cell surface markers (Patient 1 and 4: abnormal: c-$Kit^{hi}CD25^{+}$; NL: c-$Kit^{+}CD25^{-}$. Pt 2: abnormal: c-$Kit^{hi}CD25^{+}CD2^{+}$; NL:c-$Kit^{+}CD25^{-}$. Pt 3: abnormal: c-Kit^{+}; NL: c-Kit^{-}). Pt 3 had mast cell leukemia that had transformed from prior cutaneous mastocytosis and was refractory to therapy, and had higher expression of both *LIN28B* and *HMGA2* in control cells relative to other patients. K562 cells were used as a positive control for *LIN28B*. Values are expressed as fold change versus each patient's HSPCs, and displayed on a logarithmic y-axis. P values obtained by t-test.

Table I

Patients With Mast Cell Disorders

Patient	Age	Gender/race	Diagnosis	c-kit	Treatment	Outcome
1	73	M/Af-American	ASM	Wildtype	Midostaurin (PKC412)	Major Incomplete Response ; alive
2	86	F/Caucasian	ISM	D816V	Supp care H1/H2 antihistamines Prednisone	Alive
3	77	F/Caucasian	MCL	D816V	Midostaurin (PKC412)	Major Incomplete Response ; dead
4	61	F/Caucasian	ASM	D816V	Cladribine	PR; alive

The response/efficacy assessments were:

I. **Major Response**: complete resolution of at least one C-Finding and no progression of other C-Findings. C-Findings (Clinical findings) are cytopenias, osteolysis with pathologic fractures, hepatosplenomegaly and/or impaired liver function and/or ascites, and malabsorption.

- **a.** Complete response – complete disappearance of mast cell infiltrates in affected organs, decrease of tryptase levels to below 20 ng/mL, and disappearance of SM-related organomegaly
- **b.** **Incomplete response- less than 50% decrease of mast cell infiltrates in affected organs, and/or 50% decrease of tryptase levels, and /or 50% visible regression of SM-related organomegaly**
- **c.** Pure Clinical Response – without decrease in mast call infiltrates, without decrease in tryptase levels, and without regression of organomelgaly.

II. Partial Response: Incomplete regression of one or more C-Finding without complete regression and without progression in other C-Findings

- **a.** Good partial response – more than 50% regression
- **b.** Minor response – equal to or less than 50 % regression

III. No Response: C –Findings persistent or progressive

- **a.** Stable disease – C-Findings parameters show constant range
- **b.** Progressive Disease – one or more C-Findings show progression