Interrogation of EpoR Fidelity in Myelodysplastic Syndrome Hematopoiesis and Stabilization by the Immunomodulatory Agent, Lenalidomide

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Interrogation of EpoR Fidelity in Myelodysplastic Syndrome Hematopoiesis and Stabilization by the Immunomodulatory Agent, Lenalidomide

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Keywords: Erythropoietin, Lipid Rafts, Jak/Stat Signaling, E3 Ubiquitin Ligases, RNF41

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DEDICATION

This dissertation is written with dedication first to my Mom, Lynn Marie Rocha. You were all the best parts of me. I have never known anyone that was as committed, loving, and kind as you, or who worked so hard to make us happy. Although I never told you, you inspired me. Thank you for always believing in me. I miss you every single day and so much wish you were here to read this. I hope I make you proud.

Secondly, I dedicate this to my husband, Robert. Thank you for being my foundation all these years. You have been my best friend since high school. We’ve laughed together, we’ve cried together, and every hardship and achievement over the last decade (plus some) has been better because you were there. I promise to always “Keep Tight!”

And lastly, to all my friends and family, thank you for knowing I could do this. When I needed motivation, you were there. Your support and encouragement will forever be appreciated.
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The following work was made possible with the help of many, and I sincerely thank each and every one of you!
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Myelodysplastic syndromes (MDS) include a spectrum of stem cell malignancies characterized by ineffective hematopoiesis and predisposition to acute myeloid leukemia (AML) transformation. Patients are predominantly older (greater than 60 years old), with progressive cytopenias resulting from ineffective and cytologically dysplastic hematopoiesis. MDS subtypes are classified by morphologic features and bone marrow blast percentage, as well as cytogenetic pattern, as is the case for deletion 5q MDS. Interstitial deletion of the long arm of chromosome 5, del(5q), is the most common chromosomal abnormality in patients with MDS, and the 5q- syndrome, represents a distinct subset of del(5q) MDS characterized by an isolated deletion, megakaryocyte dysplasia, hypoplastic anemia, and an indolent natural history. MDS risk stratification is most commonly based on the International Prognostic Scoring System (IPSS) with survival outcomes ranging from a few months to many years based on risk factors. There are several therapeutic options for MDS including hematopoietic growth factors, immunosuppressive therapy, azanucleosides, and allogeneic stem cell transplant, however, there is still a need for more effective treatment
options, particularly targeted therapeutics. One of the most effective treatments for MDS is selective for del(5q) MDS, and is the second generation immunomodulatory agent, lenalidomide (LEN).

LEN is an analog of the known teratogen, thalidomide, and has broad biological effects including selective cytotoxicity to del(5q) clones, activation of T-cells, and expansion of erythroid precursors. In patients with del(5q) MDS, LEN is effective in up to 75% of patients, however, 50% of patients will become resistant within 2-3 years of treatment response. Studies in normal hematopoietic progenitors have shown that LEN induces expansion of the primitive erythroid precursors, which our laboratory has shown is accompanied by sensitization of progenitors to ligand induced erythropoietin receptor (EpoR) signaling. This sensitization is evidenced by increased and prolonged activation of the Signal Transducer and Activator of Transcription 5 (STAT5), compared to Epo stimulation alone. Although EpoR signaling is augmented by LEN, the exact mechanisms by which this is mediated to result in erythroid expansion are not fully characterized. In del(5q) MDS, we have shown that LEN selectively suppresses del(5q) clones via inhibition of the haploinsufficient phosphatases Cdc25c and PP2a, as well as stabilizing the human homolog of the murine double minute-2 protein (MDM2) to decrease expression of the tumor suppressor, p53, however, the mechanisms of action of LEN in non-del(5q) MDS remains elusive.

Although most anemic MDS patients have normal or elevated endogenous levels of Epo, as well as comparable levels of progenitor EpoR density relative to
healthy individuals, the biologic pathology underlying the impaired EpoR signaling in MDS is poorly defined. Recent reports have shown that membrane microdomains are important for T-cell, c-kit, and integrin signaling, however, there have been no reports on EpoR membrane localization. Lipid rafts are discrete membrane entities that provide platforms by which receptors aggregate and initiate downstream signaling. Furthermore, reports have indicated that there is a decrease in lipid raft density in GM-CSF primed MDS neutrophils, that consequently impaired production of reactive oxygen species (ROS) after fMLP stimulation, suggesting a role of rafts in MDS disease biology. Based on the role of rafts in signaling, and potential role in MDS pathogenesis, we sought to determine whether there was specific membrane localization of EpoR to the raft fractions, and whether disruption of rafts in MDS erythroids could impair EpoR signaling. To address this, we first examined the membrane localization of EpoR on the cell surface. We show here that EpoR translocates to lipid rafts in both erythroid progenitor cell lines as well as primary progenitor cells after stimulation by Epo. Furthermore, we found that Epo stimulation increases the assembly of lipid rafts, as well as the aggregation of rafts on the cell surface. Epo stimulation not only promoted the recruitment of EpoR into the raft fractions, but also downstream signaling intermediates such as Janus kinase 2 (Jak2), STAT5, and Lyn kinase. Moreover, a negative regulator of EpoR signaling, the CD45 tyrosine phosphatase, was redistributed outside of raft fractions after Epo stimulation, potentially enhancing receptor signal competence. Furthermore, disruption of lipid rafts by depletion of membrane cholesterol with MβCD (methyl-β-
cyclodextrin) inhibited EpoR signaling in both cell lines and primary bone marrow progenitor cells. Additionally, we found that inhibition of Rho-associated, coiled-coil containing protein kinase (ROCK) and/or Ras-related C3 botulinium toxin substrate 1 (Rac1), blocked the recruitment of the receptor into the raft fractions indicating a critical role of these GTPases, and associated proteins, in the transport and localization of EpoR into raft microdomains.

We next asked whether LEN could alter lipid raft assembly in erythroid precursors in the absence of Epo. LEN not only induced raft formation and aggregation but also increased F-actin polymerization. Similar to Epo stimulation, LEN alone was able to induce the recruitment of EpoR, Jak2, and STAT5 into raft fractions. Additionally, CD45 was redistributed outside of raft fractions after LEN treatment. Similarly, inhibition of ROCK blocked LEN induced raft formation and F-actin polymerization, indicating that LEN utilized effectors shared by Epo. Furthermore, LEN was able to increase raft density in raft deficient primary MDS erythroid progenitors. These data demonstrate that LEN may enhance erythroid expansion via induction of EpoR signaling competent raft platforms, to enhance survival and differentiation transcriptional response.

Recently, ribosomal protein (RP), S-14, gene \( RPS14 \) haplodeficiency was found to be a key determinant of the hypoplastic anemia in del(5q) MDS. Allelic loss of \( RPS14 \) compromises ribosome assembly, thereby causing nucleolar stress and release of free RPs that bind to and promote the degradation of MDM2, the principal negative regulator of p53. As a result, the accumulation of RPs causes lineage restricted stabilization of p53 in erythroid
precursors. Our laboratory and colleagues confirmed that cellular p53 expression levels were elevated in del(5q) erythroid precursors, and that LEN decreased expression in responding patients. However, at the time of LEN treatment failure, p53 expression was again elevated at levels exceeding those at baseline. These results suggest that LEN is initially able to reverse p53 accumulation levels and that this action may be a mechanism by which LEN is selectively cytotoxic to del(5q) clones. Subsequent studies showed that LEN inhibits the cereblon E3 ubiquitin ligase complex, the newly discovered target of LEN. Cereblon has been reported to be the principal protein involved in thalidomide induced teratogenicity. Furthermore, the cytotoxic activity of LEN in multiple myeloma is dependent on cereblon. Our laboratory found that LEN inhibits the auto-ubiquitination of MDM2, thereby stabilizing the protein, and promoting ubiquitination of and ultimately the degradation of p53. Additionally, we found that LEN blocked the binding of free ribosomal proteins to MDM2, which are liberated from the nucleosome by ribosomal stress from RPS14 haploinsufficiency, consequently stabilizing the E3-ubiquitin ligase and fostering p53 degradation.

In non-del(5q) MDS there is no cytotoxicity of MDS clones by LEN, suggesting an alternative method of erythropoiesis rescue. Although we know that LEN promotes the formation of signaling platforms, and recruitment of EpoR, we wished to determine whether there was an effect of LEN on EpoR expression, as EpoR expression is controlled through ubiquitination and proteasomal degradation. Treatment of erythroid progenitor cell lines and primary erythroid
precursors with LEN increased cellular expression of Jak2-associated EpoR in a concentration dependent manner. There was no change in mRNA expression, supporting a post transcriptional mechanism. We then investigated whether receptor up-regulation was limited to EpoR, or included other cytokine receptors. We found that LEN induced expression of another Jak2 associated Type I receptor, IL3-R, but did not alter cellular expression of c-kit, a Type II cytokine receptor. Because Type I cytokine receptor turnover is regulated by a shared E3-ubiquitin ligase, and LEN inhibited both MDM2 and cereblon, we evaluated the effects of LEN on the E3-ubiquitin ligase, Ring Finger Protein-41 (RNF41), which regulates steady state or ligand independent, Jak2 associated Type I receptor internalization. We found that LEN inhibited the ubiquitination activity of RNF41, ultimately stabilizing EpoR membrane residence and increasing expression.

In summary, MDS patients display ineffective hematopoiesis likely in part to decreased lipid raft assembly. Stimulation by Epo, or treatment by LEN, not only induced raft formation, but also induced the recruitment of both growth factor receptor, and downstream signaling intermediates into raft fractions to enhance EpoR signal fidelity. We have shown here two methods by which LEN may augment EpoR signaling. First, LEN increases lipid rafts and promotes recruitment of signaling effectors. Second, LEN increases and stabilizes the expression of EpoR through inhibition of the E3 ubiquitin ligase, RNF41. Therefore, we suggest here that LEN may have broad E3 ubiquitin ligase inhibitory effects. These data also indicate that lipid raft upregulation by LEN is
mediated through GTPases, suggesting that GTPase activation may also occur via inhibition of specific E3 ubiquitin ligases, a question to be addressed in future studies.
CHAPTER 1

Background

Myelodysplastic Syndromes (MDS)

MDS Overview. Myelodysplastic syndromes (MDS) are a heterogeneous group of stem cell disorders characterized by ineffective hematopoiesis and predisposition to acute myeloid leukemia (AML) transformation. Pathobiological features of MDS include upregulation of inflammatory response genes and corresponding cytokine production that contribute to accelerated apoptotic death of hematopoietic progenitors with consequent ineffective hematopoiesis which underlies the cytopenias characteristic of MDS.\(^1\) MDS is observed primarily in older individuals (greater than 60y) and overall survival ranges from as short as a few months to several years based on a number of disease features detailed below. Although there are instances of familial MDS, these cases are rare and have been linked to mutations in particular genes such as RUNX1.\(^2\) MDS also occurs in children, although rarely, and is often associated with constitutional genetic disorders or inherited bone marrow failure syndromes.\(^3\) The prevalence of MDS is greater in males than females, and is observed more frequently in patients previously exposed to toxic agents, such as chemotherapy or radiation.\(^4\)
Recent reports suggest that there are up to about 75 per 100,000 new MDS cases diagnosed each year in the US among individuals 65 years of age or older.\textsuperscript{5} 

**MDS classification.** Distinction of MDS subtypes utilizes the subjective morphologic characterization of cytological dysplasias that was first defined more than 30 years ago.\textsuperscript{6-8} MDS subtypes were initially characterized according to the French-American-British (FAB) classification for MDS that was developed in 1982.\textsuperscript{9,10} A diagnosis of MDS by the FAB classification was strictly based on cell morphology and bone marrow blast percentage; however, considerable prognostic overlap between subtypes and a surge of data on disease biology generated the need for a new classification system. In 2001, the World Health Organization (WHO) established a new classification that further refined subtypes, reorganized previous classifications, and added additional categories.\textsuperscript{8-11} The WHO classification was also based on morphology and blast counts, however, a single chromosomal aberration, del(5q) was introduced into the diagnostic criteria. The 2001 WHO classification implemented discrimination based on the number of lineages of cytological dysplasia and introduced the RCMD (refractory cytopenia with multilineage dysplasia) subtype, either with or without ring sideroblasts (RS). The WHO also lowered the blast threshold for AML from 30% to 20%, and moved chronic myelomonocytic leukemia (CMML) into a new category of MDS/MPN (myelodysplastic myeloproliferative neoplasm).\textsuperscript{8-10,12} Additionally, a new category was added, the 5q- syndrome, a
subtype characterized by less than 5% bone marrow blasts and an isolated chromosome 5q deletion. Detailed discussion of the 5q-syndrome will be provided in the next section.

Although the 2001 WHO was widely accepted, modest changes were recommended to this classification system in 2008. The 2008 WHO classification is still based on cellular morphology, blast percentage, and cytogenetics, but provides a more detailed subtyping system. Most notable of the revisions include reorganization of refractory cytopenias with unilineage dysplasia (RCUD) into lineage specific subtypes, extension of the description of MDS-U (unclassified), changing the 5q-syndrome to del(5q) MDS, and addition of refractory cytopenias of childhood (RCC). Undoubtedly, as new data emerges and new techniques provide more pathobiologic data, revisions will likely be needed to further elucidate distinct subtypes of MDS based upon biological drivers. A summary of the FAB and WHO classifications is provided in Table 1.

5q-Syndrome. Approximately 50% of patients with MDS carry a chromosomal abnormality. The most common chromosomal abnormality found in up to 25% of MDS patients, is interstitial deletion of part of the long arm of chromosome 5 [del(5q)]. In 1974, Van den Berghe and colleagues first described this distinct hematological subset of MDS patients and coined the term 5q-syndrome. Patients with 5q-syndrome have severe hypoplastic anemia.
Table 1. Comparison of MDS classifications according to the French-American-British (FAB) and World Health Organization (WHO) classifications.

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<tr>
<td>RA</td>
<td>1 cytopenia, &lt;5% blasts</td>
<td>RAU Unilineage dysplasia, &lt;5% blasts, &lt;15% RS</td>
<td>RCUD (RA, RN, RT) Unilineage dysplasia, &lt;5% blasts, &lt;15% RS</td>
</tr>
<tr>
<td>RARS</td>
<td>same as RA with &gt;15% RS</td>
<td>RARS Unilineage dysplasia, &lt;5% blasts, &gt;15% RS</td>
<td>RARS Unilineage dysplasia, &lt;5% blasts, &gt;15% RS</td>
</tr>
<tr>
<td>RAEB</td>
<td>cytopenia of at least 2 lineages, BM blast 5-20%</td>
<td>RCMD Dysplasia in at least 2 lineages, &lt;5% blasts</td>
<td>RCMD Dysplasia in at least 2 lineages, &lt;5% blasts</td>
</tr>
<tr>
<td>RAEB-t</td>
<td>21-30% BM blast or presence of Auer rods</td>
<td>RCMD-RS Dysplasia in at least 2 lineages, &lt;5% blasts, &gt;15% RS</td>
<td>RAEB-1 5-9% blasts, no Auer rods</td>
</tr>
<tr>
<td>CMML</td>
<td>monocytosis, up to 20% blast</td>
<td>MDS with del(5q) &lt;5% blasts, megakaryocyte dysplasia, isolated del(5q)</td>
<td>MDS-U &lt;5% blasts, dysplasia in less than 10% of any or multiple lineages</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t-MDS Therapy related</td>
<td>MDS with del(5q) &lt;5% blasts, megakaryocyte dysplasia, isolated del(5q)</td>
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<tr>
<td>RAEB-1</td>
<td>5-9% blasts, no Auer rods</td>
<td>RAEB-2 10-19% blasts, Auer rods</td>
<td>RCC &lt;5% blasts, dysplasia, pediatric</td>
</tr>
<tr>
<td>RAEB-2</td>
<td>10-19% blasts, Auer rods</td>
<td>RCMD-RS refractory cytopenia with multilineage cytopenia with ringed sideroblasts</td>
<td>RCMD-U = MDS unclassified</td>
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BM = bone marrow
RA = refractory anemia
RARS = refractory anemia with ringed sideroblasts (RS)
RAEB = refractory anemia with excess blasts
RAEB-t = refractory anemia with excess blasts with transformation
cMML = chronic myelomonocytic leukemia
RCMD = refractory cytopenia with multilineage cytopenia
RN = refractory neutropenia
RT = refractory thrombocytopenia
RCUD = refractory cytopenia with multilineage cytopenia
leading to transfusion dependence, accompanied by mild leukopenia, normal or elevated platelet counts, and atypical bone marrow megakaryocytes. In 2001, the WHO created a separate MDS subtype called the 5q- syndrome that required the presence of isolated del(5q) chromosomal abnormality and bone marrow blasts less than 5%. There is a higher prevalence of the 5q- syndrome in females compared to males (7:3) and these patients have better predicted overall survival and decreased risk for leukemic transformation. Nevertheless, overall prognosis in the 2001 category of 5q- syndrome was heterogeneous because of the inclusion of atypical cases with thrombocytopenia or neutropenia. For this reason, the WHO change the terminology to del(5q) MDS in 2008. The presence of a single cytogenetic abnormality provides the opportunity for researchers to study the pathogenesis of this one subtype of MDS, an effort often complicated by the vast heterogeneity of other subtypes. Mapping of the commonly deleted region (CDR) in the 5q- syndrome revealed an area of 1.5 megabases comprised of 41 genes located at 5q32-33. Although initial studies sought the identification of a tumor suppressor gene that could be linked to disease development, these efforts were unsuccessful. It was only recently that one specific gene in the CDR, RPS14, has been linked to the pathogenesis of the hypoplastic anemia found in del(5q) MDS. Ebert et al. performed a series of elaborate knockdown experiments, and showed that only shRNA knockdown of RPS14 was able to recapitulate the 5q- phenotype, i.e. erythroid specific proliferative arrest and apoptosis indicating its importance in the pathobiology of this disease. Haploinsufficiency for RPS14 disrupts ribosome
assembly causing ribosomal stress, and as in congenital ribosomopathies, activates p53 in erythroid precursors causing hypoplastic anemia. Further discussion of ribosomopathies and the 5q-syndrome will follow under the Ribosomopathies section of this manuscript.

**International Prognostic Scoring System (IPSS).** Although the WHO classification system takes into account morphological, cytological, and a single cytogenetic feature, it lacks other key prognostic variables, and for that reason risk stratification is accomplished primarily through the International Prognostic Scoring System (IPSS) which was published in 1997. The IPSS divides patients into four distinct categories; low risk, intermediate-I, intermediate-II, and high risk disease. Overall survival and AML transformation risk increases with risk prognostic score and category, with the median survival for untreated patients with low, Int-1, Int-2, and high risk disease of 5.7, 3.5, 1.2, and 0.4 years, respectively.

The IPSS is calculated based on three parameters; bone marrow blast percentage, number of cytopenias, and karyotype as summarized in Table 2. Each parameter is assigned a particular weighted score which are then summed to give the cumulative score representative of a particular risk category. The values for each parameter are also provided in Table 2. Although the IPSS was developed based on a data set of approximately 800 patients from the US, Europe, and Japan, the large amounts of data generated since 1997 has led some to believe a revision of the IPSS was necessary. In 2012, the revised
IPSS, IPSS-R, was published by Greenberg and colleagues. The IPSS-R was based on a significantly larger cohort of patients (IPSS, n=816; IPSS-R, n=7012) yet still utilized the same three clinical characteristics of the original IPSS, including number of cytopenias, bone marrow blast percentage, and karyotype. The major changes in the revised system include revised bone marrow blast percentage categories, grading the severity of each cytopenia, expansion of chromosomal abnormalities captured, and an increase in the diagnostic parameters taken into account, such as age, serum ferritin, and lactate dehydrogenase (LDH). Notably, the IPSS-R has 5 risk categories and 5 cytogenetic risk categories as opposed to four and three in the original IPSS, respectively. The IPSS-R was published in September of 2012; however, at the time of this manuscript, the IPSS is still the most commonly used prognostic scoring system by clinicians. Although, the IPSS is the most commonly used system, it should be noted that there are other proposed risk stratification systems for MDS such as the WHO classification-based Prognostic Scoring System (WPSS). The WPSS takes into account WHO morphologic subtypes as well as transfusion dependence, and consists of 5 distinct risk categories including very low, low, intermediate, high and very high risk categories. The most notable characteristic of the WPSS is the effectiveness of determining prognosis at any time point during the disease, allowing for real time treatment decision making at any time in the disease natural history. Yet another scoring model published in 2008, is the MD Anderson Prognostic Scoring System (MPSS). A major component of this system is that disease duration and
## International Prognostic Scoring System (IPSS) of MDS

<table>
<thead>
<tr>
<th>Score</th>
<th>LOW</th>
<th>0.5-1.0</th>
<th>1.5-2.0</th>
<th>2.5+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Survival (years)</td>
<td>5.7</td>
<td>3.5</td>
<td>1.2</td>
<td>0.4</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Score Value</th>
<th>Good</th>
<th>Intermediate</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td>1 abn</td>
<td>2+ abn</td>
</tr>
<tr>
<td>0.5</td>
<td>del(5q)</td>
<td>del(5q) + 1 abn</td>
<td>chr. 7 abn</td>
</tr>
<tr>
<td>1</td>
<td>del(20q)</td>
<td>del(20q) + 1 abn</td>
<td>-Y + 1 abn</td>
</tr>
</tbody>
</table>

- **BM Blast %**: <5% | 5-10% | >10%
- **No. Cytopenias**: 0/1 | 2/3 | -Y
- **Karyotype**: Good | Intermediate | High

**Cytopenias**: Hemoglobin <10g/dL, Neutrophils <1.8k/µL, Platelets <100k/µL

**Karyotype**
- **Good**: Normal
- **Intermediate**: 1 abn
- **High**: 2+ abn

**Abnormalities**: abn: abnormality
previous therapies are taken into account, however, it does not consider bone marrow blast percentage (below 30%) or WHO classification.\textsuperscript{30,31}

Although there are several scoring systems available for risk stratification in MDS, reports continue describing novel prognostic variables. Some such variables include $\beta$2-microglobulin, bone marrow fibrosis, hypoalbuminemia, and others.\textsuperscript{32} It should also be noted that some of these systems, in particular the IPSS, are limited in their effectiveness due to the heterogeneity within each subgroup, particularly with respect to the high proportion of normal karyotype assessed by metaphase cytogenetics.\textsuperscript{33} Currently, new molecular biomarker approaches are being proposed including use of single nucleotide polymorphism (SNP) arrays (SNP-A) or exome sequencing to identify very small genetic lesions not resolved by metaphase cytogenetics.\textsuperscript{33} It reasons then, that these scoring systems will continue to evolve to better delineate risk based on biological features.

**MDS treatment.** Often the first treatment for management of anemia in patients with MDS, particularly lower risk MDS, is the use of erythroid stimulating agents (ESAs) such as recombinant erythropoietin (Epo), either alone or in combination with granulocyte/macrophage colony stimulating factor (GM-CSF), or granulocyte colony stimulating factor (G-CSF).\textsuperscript{4,34} The use of ESAs grew in popularity in the early nineties; however, response rates were not robust. Initial reports of erythroid response rates to Epo ranged from about 10-20%.\textsuperscript{34,35} With development of response predictive models, the rates of response to Epo have
risen with proper patient selection, however, they remain relatively low (30-60%) and differ greatly between studies and institutions. A relatively new approach to treating myelodysplastic syndromes is the use of immunosuppressive therapies (IST) such as cyclosporine, and ATG (anti-thymocyte globulin). These treatments have had greater success inducing effective hematopoiesis in younger patients with lower risk disease, however, response rates again vary greatly from center to center. Current studies are underway trying to delineate those patients expected to respond to IST based on CD4/CD8 ratios or other biomarkers.

Another class of MDS therapeutics, the azanucleosides, includes two of the three currently Food and Drug Administration (FDA) approved therapies for MDS, azacitidine and decitabine. The azanucleosides are believed to target the DNA hypermethylation observed in MDS patients. This treatment option is primarily used in higher risk patients, or those who fail primary therapies. Current studies are underway testing novel azanucleosides including the use of new orally available compounds and combination trials. The third of the FDA approved drugs for MDS, is lenalidomide (LEN) (Revlimid®, Celgene Corporation, Summit, NJ). LEN is approved for the treatment of red blood cell transfusion dependent patients with lower risk MDS and a chromosome 5q deletion. It is the first cytogenetically targeted therapy for MDS and will be discussed in greater detail in the next section of this manuscript.

The only potentially curative option currently for the treatment of MDS is allogeneic stem cell transplant (SCT). This is often not an alternative for most
patients due to advanced age at disease diagnosis, co-morbidities, or lack of a compatible donor. Encouraging though, is the number of clinical trials either currently ongoing or in preliminary phases on novel targeted approaches to MDS. Some of these trials include inhibitors of transforming growth factor \( \beta \) (TGF-\( \beta \)), an inhibitory hematopoietic cytokine family; indoleamine 2,3 dioxygenase (IDO) inhibitors that abrogate expansion of T\(_{\text{reg}}\) and myeloid derived suppressor cells (MDSC); multi-kinase inhibitors that block PI3K and Akt pathways; aminopeptidase inhibitors which are amino acid recycling regulators; and p53 inhibition, particularly in LEN-resistant del(5q) MDS.\(^{37}\)

**Immunomodulatory drugs (IMiDs®)**

**Thalidomide.** Thalidomide is a glutamic acid analogue that was developed in Germany in 1954 (Figure 1).\(^{39,40}\) Thalidomide was approved in Europe as a sedative and anti-emetic later that decade under relatively lax regulatory scrutiny.\(^{39-41}\) Minimal animal models were tested, and similarities to other barbituates led governing boards to conclude its safety as a sedative.\(^{42}\) Unfortunately, only a few years later in 1961, the drug was withdrawn from the market due to a tragically high number of cases of teratogenicity in newborn babies.\(^{39-41}\) It has been reported that more than 10,000 children from 46 countries were affected worldwide.\(^{41,42}\) The majority of these cases involved limb malformations and congenital defects.\(^{41}\) Although the use of thalidomide as a sedative was encouraging, particularly due to the lack of toxicity recorded after
overdose, the US FDA never approved the drug due to unanswered concerns regarding the drug’s safety, particularly with respect to neurotoxicity.\textsuperscript{39,41}

Interestingly, the European animal models used to test the efficacy and safety of thalidomide included only mice and rodents, however, it was later found that the teratogenicity of thalidomide is species specific and is restricted to chickens, rabbits, and humans.\textsuperscript{42} Even after several decades of research, the exact mechanisms of the teratogenicity remains unclear, although recent studies implicate inhibition of the cereblon E3-ubiquitin ligase complex.\textsuperscript{39,43} Cereblon will be discussed in further detail under the \textit{E3-ubiquitin ligase} section of this manuscript.

A diverse array of biological effects has been attributed to thalidomide. Some of these include anti-inflammatory activity, T and NK cell activation, and suppression of tumor necrosis factor $\alpha$ (TNF$\alpha$) elaboration.\textsuperscript{41,42,44} These attributes, as well as others, led thalidomide to be termed the first immunomodulatory agent (IMiD$\textsuperscript{®}$) in clinical use. The anti-inflammatory effects
were found to be extraordinarily effective for erythema nodosum leprosum (ENL) and in 1998 the FDA approved the use of thalidomide in ENL patients.\textsuperscript{40,42,44} Further studies with thalidomide revealed that it was also an effective anti-angiogenic agent, a potentially useful attribute for treating cancer.\textsuperscript{40,42,44} In 2006, after several successful clinical trials, the FDA approved thalidomide for the treatment of multiple myeloma (MM).\textsuperscript{42,44} Although thalidomide is now approved for the treatment of several conditions, its use is heavily regulated. In order to be prescribed in the US, patients, physicians, and pharmacists must all be registered in the System for Thalidomide Education and Prescription Safety (STEPS) program. This program was created by Celgene (Summit, NJ) in order to limit both the marketing and the adverse effects of the compound observed worldwide.\textsuperscript{42,44}

\textbf{Lenalidomide.} Studies of second generation IMiDs\textsuperscript{®} were met with great optimism over the last decade. These compounds are less toxic and more potent than their parent compound.\textsuperscript{44} Lenalidomide (LEN, Revlimid\textsuperscript{TM}, CC-5013) is the most well studied second generation IMiD\textsuperscript{®} and is structurally similar to thalidomide with only the addition of an amine group and loss of one carbonyl group (Figure 1). LEN is up to 50,000 fold more potent than thalidomide as measured by inhibition of TNF-\(\alpha\), and more effectively suppresses elaboration of the pro-inflammatory cytokines IL-1\(\beta\), IL-1, IL-6 and others, while promoting the production of anti-inflammatory cytokines such as IL-10.\textsuperscript{42,45,46} Additionally, LEN also enhances NK cell activity by promoting population expansion, stimulates
activation of T-cells by increased production of IL-2 and IFN-γ, and is 3 times more potent than thalidomide in its ability to inhibit angiogenic response.$^{45,46}$ The modulation of the immune system by LEN is an intriguing therapeutic option for solid tumors. Often, these tumors are able to evade the immune system by escaping activation of CD4+ cytotoxic T-cells and CD8+ helper T-cells.$^{45}$ LEN activates both CD4+ and CD8+ cells, as well as hyper-sensitizes the T-cell receptor (TCR), and thus may be effective at limiting solid tumor burden.$^{45}$ Tested as both a single agent and in combination in a number of solid malignancies including melanoma, prostate, pancreatic, thyroid, brain, ovarian cancer, and others, efficacy in solid tumors is thus far disappointing.$^{45}$ However, there are still more than 200 clinical trials ongoing and as the mechanisms of LEN are uncovered, it is likely that these findings may prove more positive.$^{45}$

Although, LEN’s activity in solid tumors has not been particularly encouraging, there is considerable efficacy in hematological malignancies. In 2005, combination treatments of LEN with the steroids prednisone or dexamethasone, with or without the addition of the proteasomal inhibitor, bortezomib, or in combination with the standard care chemotherapy, melphalan, in MM patients received much attention.$^{40,46}$ These combination therapies were very successful, in fact, in the Phase II LEN-melphalan-prednisone trial, there was partial response or better in over 80% of elderly patients newly diagnosed with MM.$^{40}$ Additionally, there was almost a 25% complete response rate.$^{40}$ These data were staggering considering the overall poor success of previous regimens. In 2006, LEN was approved for the treatment of MM in patients with
one prior therapy (www.fda.gov). Although these data are particularly hopeful, perhaps the most successful story of LEN is in the treatment of del(5q) MDS.

**Lenalidomide in MDS.** In 2005, a clinical study of 43 lower risk MDS patients, with either no response to Epo or limited suspected benefit was published by List et al. in the New England Journal of Medicine. In this study, 56% of all patients responded with almost half achieving sustained transfusion independence. Perhaps the most intriguing aspect of this study, was the fact that 83% of the patients with a del(5q) abnormality responded with normalization of hemoglobin. These numbers were shocking as previous therapies for MDS, particularly del(5q) MDS, had been disappointing. In a follow up study of 148 del(5q) MDS patients, 76% showed reduced need for transfusions, while 67% became transfusion independent. Transfusion independence was accompanied by cytogenetic improvement suggesting cytotoxicity to the del(5q) clone. These studies prompted the FDA to approve the treatment of LEN in lower risk del(5q) MDS patients in 2005. As exciting as these results were, and continue to be, the exact mechanisms and direct targets of LEN were, and are, still not clear. We do, however, know that LEN has specific mechanisms of action that can account for the biological effects of LEN in non-del(5q) and del(5q) MDS independently.

**Lenalidomide in non-del(5q) MDS.** In non-del(5q) MDS, LEN restores hematopoiesis by expanding the erythroid progenitor population, as well as
overcoming the diminished Epo induced STAT5 activation in MDS patients without observed cytotoxicity to the MDS clone.\textsuperscript{47-49} Although MDS patients have either comparable or elevated levels of endogenous Epo with no defects in ligand binding to receptor, activation of STAT5 through the Epo receptor (EpoR) is diminished compared to normal progenitors.\textsuperscript{49} LEN augments Epo induced STAT5 activation and prolongs the duration of activation compared to Epo stimulation alone, promoting erythroid expansion. Although LEN is able to rescue ineffective hematopoiesis in non-del(5q) MDS patients, the transfusion independence response rate for this subset of patients is only about 25\%.\textsuperscript{6} In 2008, Ebert et al. published a gene signature which was predictive for LEN response in non-del(5q) MDS and found that there was a decrease in a set of erythroid differentiation genes in patients that responded to LEN.\textsuperscript{50} Upon LEN treatment, these genes were upregulated correcting the defective erythroid differentiation patterns.\textsuperscript{50} Although this gene signature may provide a good biomarker to identify the non-del(5q) MDS patients that may respond to LEN, clinical use of this data has yet to occur. Furthermore, underexpression of these lineage specific genes may reflect the impairment in EpoR signaling. The precise mechanism of action of how erythroid expansion, or induction of erythroid differentiation genes occurs, is not yet fully understood and is addressed in this study.

\textbf{Lenalidomide in del(5q) MDS.} Response rates in del(5q) MDS are much more promising but seem to occur via mechanisms that are distinct from that in
non-del(5q) MDS. There is approximately a 75% transfusion response rate in lower risk non-del(5q) patients treated with LEN.\(^1\) These astonishing findings led to LEN becoming the first targeted therapy for lower risk del(5q) MDS patients, approved by the FDA in 2005. It was also observed that responding patients commonly showed loss of the del(5q) clone after treatment, suggesting direct cytotoxicity to this clone.\(^1\) Selective cytotoxicity of the del(5q) clone was confirmed by our laboratory with collaborators in 2009.\(^5\) We determined that clonal sensitivity was a result of LEN’s inhibitory effect on two haplodeficient phosphatases, CDC25c and PP2Ac.\(^5\) Both genes are located in the del(5q) CDR, and when non-del(5q) MDS primary samples were lentivirally infected with shRNA for either CDC25c and/or PP2A, the cells underwent an apoptotic response after LEN exposure similar to del(5q) cells.\(^5\) We also found the LEN inhibited the enzymatic activity of CDC25c and PP2Ac, by direct and indirect actions, respectively.\(^5\) These phosphatases are key regulators of the cell cycle, and in our experiments, LEN induced a G2-M cell cycle arrest in the del(5q) cells.\(^5\) These results were consistent with a previous study showing selective cytotoxicity of the del(5q) clone to LEN. In that study, the authors proposed the selective cytotoxicity may involve upregulation of the CDR encoded tumor suppressor gene, \textit{SPARC}.\(^2\) Upregulation of SPARC by LEN however was not restricted to del(5q) clones, and therefore appears to be universal drug effect.\(^2\) In summary, LEN enhances erythropoiesis in both non-del(5q) and del(5q) MDS, albeit by two different mechanisms. Erythroid differentiation and expansion is observed in non-del(5q) patients, whereas, selective cytotoxicity is observed in
del(5q) patients. Additional findings from our laboratory on the mechanisms of LEN in del(5q) patients will be discussed *E3 ubiquitin ligase* section of this manuscript.

**Pomalidomide.** Pomalidomide (CC-4047, Actimid, PomaLyst, Celgene Corporation, Summit, NJ) is a third generation immunomodulatory agent, that may be even more potent than LEN. Similar to LEN and thalidomide, pomalidomide suppresses TNF-α, stimulates T-cells, expands NK cell numbers, and has anti-angiogenic properties. Currently there are several clinical trials investigating the effects of pomalidomide, particularly in MM.\(^ {40}\) The first Phase I study of pomalidomide in multiple myeloma, showed a reduction in paraprotein in 67% of patients and a complete response in 10% of patients.\(^ {40}\) Subsequently, several Phase II studies were performed testing the efficacy of dexamethasone and pomalidomide in refractory or relapsed MM. Results were very encouraging with responses observed in more than 60% of patients.\(^ {40,53}\) Perhaps of most importance, is the effectiveness of pomalidomide/dexamethasone in thalidomide, LEN, and bortezomib refractory multiple myeloma patients.\(^ {40,53}\) In one study, pomalidomide/dexamethasone treatment was effective in 40% (8/20) of LEN-refractory patients, 37% (6/16) of thalidomide refractory patients, and 60% (6/10) of bortezomib refractory patients as well as 60% (3/5) bortezomib and LEN refractory patients.\(^ {53}\) These surprising results, in combination with several other clinical studies, led to the FDA accelerated approval of pomalidomide in refractory myeloma patients on Feb 8, 2013 (www.fda.gov). Currently,
pomalidomide is also being testing in myeloproliferative neoplasms and in some solid tumors with efficacy and reporting yet to be concluded.

Ribosomopathies

**Overview.** In 1999, mutations in ribosomal protein S19 gene (*RPS19*) were found to be associated with the disease pathogenesis of Diamond-Blackfan anemia (DBA). Although it may have not been known at the time, this finding would become the premise for the characterization of a spectrum of disorders now known as **ribosomopathies**. Several congenital hypoplastic anemias caused by mutations of genes or somatic deletion of genes encoding proteins involved in ribosome biogenesis as in del(5q) MDS, can be broadly categorized as ribosomopathies. In order to understand these diseases, a brief review of ribosome biogenesis is warranted (Figure 2). The creation of ribosomes is an energy intensive process that is highly regulated and is vital to both cell growth and cell division. This process involves hundreds of individual steps, 4 ribosomal RNAs (rRNA), at least 80 different ribosomal proteins (RPs), over 150 associated proteins, and an estimated 70 small nucleolar RNAs, as well as the coordinated effort of RNAses. Ribosomal DNA is transcribed into a 45S precursor by RNA polymerase (pol) I. This precursor will be spliced into 28S, 18S, and 5.8S rRNAs. At the same time, RNA pol III is transcribing an additional 5S subunit. In association with RPs transcribed by RNA pol II, and other associated proteins, 60S and 40S ribosomal subunits will be formed from
these precursors, and will be exported to the cytoplasm where they join to form
the mature 80S ribosome (Figure 2).\textsuperscript{56,58} Ultimately, it is the responsibility of the
ribosome to translate mRNA and manufacture all proteins necessary for the life
of the cell. These process are highly regulated by a stoichiometric relationship
between precursors and mature subunits, and deregulation can have severe
cellular consequences.\textsuperscript{56}

The finding of \textit{RPS19} mutations in DBA provided the foundation for the
study of a number of disorders now known as ribosomopathies. Interestingly, the
majority of these disorders involve bone marrow failure associated with
hypoplastic or non-regenerative anemia. DBA is a heterogeneous disorder
characterized by anemia, erythroid failure, congenital abnormalities, and an
increased risk for leukemic transformation.\textsuperscript{54,56,59} DBA is usually detected early in
life (within the first year) and is a classic bone marrow failure syndrome with
ineffective hematopoiesis and increased apoptosis of progenitors in the bone
marrow compartment.\textsuperscript{56,59} Mutations in \textit{RPS19} occur in approximately 25% of all
DBA patients, and since the original publications, mutations in four other RP
genes have been identified accounting for up to 50% of mutations detected DBA
patients.\textsuperscript{58,59} \textit{RPS19} is one of the RPs involved in the creation of the 40S subunit
from the 18S subunit, and DBA patients harboring either mutations or
haplodeficiency for this gene have decreased levels of the 40S subunit.\textsuperscript{56}
However, the exact mechanisms by which \textit{RPS19} causes a defect in 40S
assembly is yet to be clearly defined.\textsuperscript{56}
Figure 2. Overview of ribosome biogenesis and ribosomopathy. Ribosome biogenesis and suspected locations of altered biogenesis in ribosomopathies. Some of the suspected genes involved are provided in italics. (DC) dyskeratosis congenital, (CHH) cartilage-hair hypoplasia, (TCS) Treacher-Collins syndrome, (SDS) Shwachman-Diamond syndrome, (DBA) Diamond-Blackfan anemia.

A second bone marrow failure syndrome caused by defects in ribosome biogenesis is Shwachman-Diamond syndrome (SDS). Nearly all SDS patients have severe neutropenia and anemia, with pancytopenia is observed in up to 65% of patients.\textsuperscript{56,57} In SDS, mutations in the \textit{SBDS} gene are suspected to be involved in disease pathogenesis. \textit{SBDS} is also suspected to be involved in 40S maturation, although the mechanism by which this occurs in humans has yet to be determined.\textsuperscript{57} In X-linked dyskeratosis congenital (DC), mutations in \textit{DKC1}, a
gene thought to be involved in the processing and modifications of the 45S subunit, are suspected to be linked to disease pathogenesis.\textsuperscript{56,57} Pancytopenia and severe aplastic anemia is observed in the majority of X-linked DC patients.\textsuperscript{56,57} Cartilage hair hypoplasia (CHH), along with several other clinical features, presents with hypoplastic anemia and is suspected to be caused by mutations in the \textit{RMRP} gene, which is important for the RNAse complex and splicing of the 45S subunit.\textsuperscript{56,57} Treacher-Collins syndrome (TCS), characterized by craniofacial abnormalities similar to those observed in DBA, is thought to be caused by mutations in \textit{TOCF1}. The \textit{TOCF1} gene encodes Treacle which effects ribosome DNA transcription by binding an RNA pol I transcription factor.\textsuperscript{57,58} Collectively, all these syndromes represent classical ribosomopathies and bone marrow failure, suggesting a critical role for ribosome biogenesis in the bone marrow compartment.

\textbf{The 5q- syndrome as a ribosomopathy.} In 2008, Ebert et al. demonstrated that haploinsufficiency for \textit{RPS14} was responsible for the hypoplastic anemia of the 5q- syndrome.\textsuperscript{23} Although, haploinsufficiency for \textit{CDC25c} and \textit{PP2Aca} has been demonstrated to be responsible for LEN sensitivity,\textsuperscript{51} these \textit{RPS14} data were the first to describe a pathway singularly responsible for the pathogenesis of anemia in MDS. The CDR of del(5q) has been extensively studied, and most research sought to find a tumor suppressor gene responsible for disease development. These studies were all unsuccessful at identifying such a gene.\textsuperscript{20,22} Since no mutations or biallelic deletions have
ever been identified, Ebert et al. hypothesized that haploinsufficiency may be the cause of disease phenotype. In an effort to test this hypothesis, the investigators generated a panel of shRNAs targeting the 41 genes contained within the CDR. Each gene was knocked down by approximately 50% in order to mimic the allelic haploinsufficiency. Partial loss of only \textit{RPS14} mimicked a 5q-phenotype, which was resolved in \textit{RPS14} add back experiments. \textit{RPS14} knockdown caused proliferative arrest and loss of differentiation of mature erythroid cells, and ultimately is the cause of hypoplastic anemia in the 5q-syndrome phenotype. Although the exact functions of \textit{RPS14} are unknown, knockdown resulted in a decrease in 18S rRNA suggesting aberrant splicing of precursor RNAs. These data were confirmed in a mouse model, where deletion of all genes in the human CDR including \textit{RPS14} recapitulated the disease. In summary, \textit{RPS14} haplodeficiency is implicated in del(5q) MDS pathogenesis, and provides further insight into disease biology.

**Phenotypical differences in ribosomopathies.** These syndromes all represent a new class of syndromes known as ribosomopathies, and interestingly, most are also bone marrow failure syndromes. For the first time, a group of diseases is recognized by the failure of a cell to effectively produce mature functioning ribosomes, whether it be due to mutation or haplosufficiency. The question arises, what causes the distinct phenotypes in each of these diseases, and why do they seem to be specific or preferential to the bone marrow? The answer to this is yet to be determined; however, proposed models exist and are
currently being investigated. According to a review by Ganapathi and Shimamura, these models are generally as follows, 1) highly proliferative cells such as erythroid progenitors and/or hematopoietic stem cells are particularly sensitive to ribosome biogenesis ineffectiveness, 2) specific mRNAs particular to the bone marrow may be selectively effected, 3) certain tissues may be selectively sensitive to decreased ribosome biogenesis, 4) cells with high output of RPs may be selectively sensitive to an accumulation of free RPs in the cell, and 5) increased cell cycle arrest and/or apoptosis may be particularly detrimental to hematopoietic stem cells.\(^\text{56}\) Although, none of these models have yet to be proven, there is one common theme that may serve to link them, and describe how aberrations in ribosome biogenesis pathway may cause a number of phenotypically diverse syndromes. This link is an accumulation of the tumor suppressor, p53.

**Stabilization of P53.** The p53 tumor suppressor has a number of cell regulatory functions including cell cycle and apoptosis. The E3-ubiquitin ligase, MDM2, is the principal negative regulator of p53 and targets p53 for proteasomal degradation. Free or unbound RPs are able to bind to MDM2 rendering it unable to bind to, and ubiquitinate p53, thus leading to the stabilization of p53.\(^\text{60}\) In ribosomopathies, disruption of ribosome biogenesis results in nucleolar stress and the release of free, unbound RPs. These RPs bind to and promote the degradation of MDM2, thereby stabilizing p53 (Figure 3).\(^\text{60,61}\) Haploinsufficiency of *RPS14* and *RPS19* resulted in erythroid specific accumulation of p53 and
consequent cell cycle arrest. This phenomenon likely accounts for the lack of mature erythroid cells in both the 5q- syndrome and DBA. Interestingly, inhibition of p53 restored hematopoiesis, while activation by Nutlin-3 also impaired erythropoiesis. Similarly, in Barlow’s et al. syntenic mouse model, phenotypically similar to the 5q- syndrome, inactivation of p53 was sufficient to rescue the hematologic phenotype in these animals. These findings suggest that lineage specific stabilization of p53 due to nucleolar stress and excessive unbound RPs, may account for the phenotypic heterogeneity of bone marrow failure syndromes and/or ribosomopathies and may be a potential therapeutic target of these syndromes.

Figure 3. Stabilization of p53 after ribosomal or nucleolar stress observed in ribosomopathies. Without nucleolar stress, the 40S and 60S with ribosomal proteins (RP) come together to form mature, functioning ribosomes, and MDM2 is free to bind and ubiquitinate p53. However, under nucleolar stress conditions, unbound RPs are able to bind to MDM2 preventing its binding to p53 resulting in stabilization of p53. (rRNA) ribosomal RNA.
Hematopoiesis

Overview. Hematopoiesis, or the process of mature blood cell production, is a highly regulated process that in addition to producing the cells needed to maintain vital functions, is also regulated in times of infection or bleeding and is often deregulated in hematological malignancies. Hematopoiesis occurs through an intricate coordination between cytokines and transcription factors.\textsuperscript{63-65} Although a full description of the complexity of hematopoiesis may very well encompass this entire manuscript, a brief review is warranted for the following study. Briefly, hematopoietic stem cells (HSC), capable of both self-renewal and commitment to all mature blood lineages, differentiate into lineage committed progenitors which mature into precursors ultimately resulting in blood cells.\textsuperscript{63-65}

Upon particular cytokine stimulation, multipotent HSC will differentiate into either common lymphoid (CLP) or common myeloid progenitors (CMP).\textsuperscript{63-65} CLPs will mature into T-cell and natural killer (NK) cell progenitors, which turn to mature T- and NK cells.\textsuperscript{63-65} The CLP may also differentiate into a B-cell precursor, and ultimately mature B-cells.\textsuperscript{63-65} The CMP will differentiate into mature erythrocytes, megakaryocytes and platelets, neutrophils, macrophages, basophils, eosinophils, and mast cells.\textsuperscript{63-65} The progenitor and differentiating status of primary samples can be identified \textit{in vitro} using colony forming capacity (CFA) assays. In CFAs, primary cells are plated in a semi-solid medium supplemented with appropriate cytokines, and after 14 days, colony types, or progenitor potential, may be determined based on the different morphological characteristics of the
Colonies are the product of colony forming units-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) is a mixed lineage progenitor that differentiates into CFU-granulocyte, macrophage progenitors (CFU-GM), as well as the burst forming unit-erythroid (BFU-E). BFU-E are the most primitive of the erythroid progenitors and are dependent on the growth factor, erythropoietin (Epo), for maturation. BFU-E will differentiate into CFU-E which are also dependent on Epo for maturation, and will eventually complete terminal maturation into erythrocytes. The maturation and proliferation of the erythroid lineage is also dependent on the transcription factor, GATA-1. A decrease in GATA-1 expression in a mouse model resulted in a decrease in erythroid precursors. A thorough review of the erythropoietin receptor signaling pathway and the transcription factors involved in erythropoiesis will be provided under Erythropoietin Receptor Signaling. An overview of hematopoietic differentiation is represented in Figure 4.

**Hematopoiesis in MDS.** A hallmark of all bone marrow failure syndromes is ineffective hematopoiesis. Erythropoiesis is often highly disrupted in MDS despite comparative levels of the EpoR, and similar or elevated levels of endogenous Epo in MDS patients compared to normal controls. In the case of del(5q) MDS, defective erythropoiesis is caused by allelic haploinsufficiency for the RPS14 gene product and consequent lineage specific stabilization of p53. Alternatively, in non-del(5q) MDS, there are several factors contributing to ineffective hematopoiesis and erythropoiesis. Kitagawa et al. demonstrated...
Figure 4. Hematopoiesis. The hematopoietic stem cell (HSC) may differentiate into either common lymphoid or common myeloid progenitors (CLP or CMP). The CLP produces all mature T-, B-, and NK cells, while the CMP will differentiate into all other blood cell types. The colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), matures into CFU-granulocyte, macrophage progenitors (CFU-GM) that will mature to neutrophils and macrophages, as well as the burst forming unit-erythroid (BFU-E), and colony forming unit-erythroid (CFU-E), that will eventually evolve into mature erythrocytes.

that bone marrow macrophages from MDS patients express higher levels of tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), inhibitory hematopoietic cytokines, compared to normal primary cells. An increase in these cytokines suppresses maturation and differentiation in normal hematopoietic progenitors, and is implicated in the observed cytopenias in
MDS. These inflammatory cytokines also induce surface membrane expression of the death receptor, Fas, and its ligand, Fas-L, in erythroids enabling lineage specific apoptosis. Alternatively, defective hematopoiesis in MDS may also relate to epigenetic silencing of genes critical to cell growth and maturation. Aberrant promoter methylation of genes involved in cell cycle regulation, differentiation, and apoptosis. Hypermethylation of key regulatory genes leading to gene silencing and decreased expression of proteins such as survivin, CHK2, and WT1, lowers cellular threshold for apoptosis in early progenitors thereby accelerating the loss of maturing erythroid cells. Wei et al. used a CHIP-Seq analysis in CD34+ cells isolated from MDS patients to identify hypermethylation involving 36 genes, the majority of which were involved in NF-κB activation and innate immunity. Del Rey and colleagues showed that hypermethylation silencing key genes involved in cell survival, i.e. Bcl2 and ETS. Del Rey et al. also showed aberrant innate immune response due to hypermethylation and silencing of IL27RA and DICER1, regulators of microRNA biogenesis. Additionally, effectors of DNA methylation such as DNA methyltransferase (DNMT)-3a and 3b, were found to be increased in higher risk MDS patients compared to lower risk patients and normal controls, providing further evidence that gene methylation and epigenetic gene silencing in general may be important in the pathogenesis of the disease. These findings provided the basis for 2 of the 3 of the drugs currently approved by the FDA for MDS, 5-azacitidine (azacitidine, Vidaza®) and 5-aza-2'-deoxycytidine (decitabine, Dacogen®). These azanucleosides were shown to have in vivo
demethylating properties and improve hematopoiesis as evidenced by hematologic improvement in 30-50% of MDS patients.\textsuperscript{74,75}

In the following investigations, focus will be placed on ineffective erythropoiesis in MDS, particularly as it relates to Epo initiated signal response in MDS progenitors.

**Erythropoietin Receptor Signaling**

**Erythropoietin.** Erythropoietin (Epo) is a 34kD glycoprotein hormone that is responsible for red blood cell (RBC) survival, proliferation, and differentiation.\textsuperscript{70,76-78} The Epo gene was cloned in 1985 following delineation of gene location and subsequent functional analysis.\textsuperscript{70,76-79} Epo is primarily produced in the kidney with about 20% production from the liver in adults.\textsuperscript{77,79} The Epo gene is transcriptionally regulated and is activated in times of hypoxia or bleeding, in addition to balancing the basal level of mature erythrocyte mass.\textsuperscript{11} Recombinant human Epo (rhEpo) has been used to treat the anemia in conditions of endogenous hormone production such as renal failure and in acquired immunodeficiency syndrome (AIDS), as well as in patients with a variety of causes of anemia such as that related to chemotherapy, and in patients with MDS.\textsuperscript{70,76,77,79} In each of these cases, treatment with rhEpo induces the production of mature RBCs, alleviating anemia in a significant proportion of patients. Interestingly, due to the increase in RBC production and tissue oxygen
delivery, Epo has been exploited as a doping agent in endurance related sports, and has been banned in most countries by sporting governing agencies.\textsuperscript{79}

**Erythropoietin receptor.** All biological effects of Epo occur after binding of the cytokine to the cognate dimerized receptor, EpoR. The primary cell lineage expressing EpoR is erythroid progenitors, although its expression has been identified on a number of cell types including endothelial cells, mammary, brain, kidney and cardiac muscle cells, and may be found on a number of non-hematologic tumor types.\textsuperscript{80-82} The EpoR is a Type I cytokine receptor with no intrinsic kinase activity. Although it is responsible for all Epo induced cell stimulation, there are predicted to be less than 1000 receptors per cell.\textsuperscript{70,79,83,84} This finding suggests that the regulation of its production, and cell surface expression, is a highly regulated process.

**EpoR maturation, transport, and turnover.** Since the cloning of Epo and subsequently its receptor, the maturation, transport, and turnover of the receptor has been extensively investigated. Cell surface expression of the EpoR is dependent on the Janus Kinase 2 (Jak2) protein.\textsuperscript{70} EpoR is initially contained in the endoplasmic reticulum (ER) where only about 20\% will mature and be transported to the Golgi apparatus.\textsuperscript{83} Of the Golgi EpoR, only a small proportion will fully mature and be transported to and expressed on the cell surface.\textsuperscript{83} This demonstrates the highly regulated process of cellular EpoR expression and assures that an excess of receptor is readily available under conditions of
hypoxia and/or bleeding for the production of mature RBCs. Jak2 binds to the EpoR in the ER and with assistance of an EndoH oligosaccharide, is transported to the Golgi apparatus as a receptor complex.\textsuperscript{83} Full maturation of the receptor and transport to the cell surface occurs after heavy glycosylation.\textsuperscript{83} Upon receptor stimulation by its ligand, EpoR is ubiquitinated at the cell surface and is quickly internalized into the cytoplasm. EpoR ubiquitination targets the protein for proteasomal degradation which prevents further signal transduction by removing the phosphorylated tyrosine residues on the cytoplasmic tail, and allows parts of the receptor to be recycled.\textsuperscript{84-86} Two E3 ubiquitin ligases have been implicated in the degradation of EpoR and Type I Jak2-associated cytokine receptors, β-Trcp and RNF41.\textsuperscript{85,87} E3 ubiquitin ligases and their functions as they relate to these investigations will be discussed in the \textit{E3 Ubiquitin Ligase} section.

**EpoR signaling.** Structure of the EpoR is similar to other Type I cytokine receptors including a cytoplasmic, transmembrane, and extracellular domain and no intrinsic kinase activity.\textsuperscript{78,88,89} The signaling cascade triggered by EpoR stimulation has been extensively studied, and a generalized schematic is summarized in Figure 4. Upon Epo binding to its receptor, a conformational change of the receptor causes homodimerization.\textsuperscript{79,88} Dimerization causes the phosphorylation and activation of the constitutively associated Jak2 protein.\textsuperscript{70,78,79,88,90} Mouse embryos without Jak2 lack RBCs.\textsuperscript{78} Jak2 autophosphorylates itself but also phosphorylates 8 tyrosine residues on the
Figure 5. Schematic of EpoR Signaling. Erythropoietin binds EpoR which homodimerizes and triggers auto-phosphorylation of Jak2. Jak2 phosphorylates tyrosine residues on the cytoplasmic tail of EpoR as well as STAT5, the latter of which then dimerizes and translocates into the nucleus where it binds DNA and turns on pro-survival, proliferation, and differentiation genes. The transmembrane tyrosine phosphatase CD45 is a negative regulator of the pathway, which dephosphorylates tyrosine residues on Jak2, lyn kinase, and the EpoR cytoplasmic tail.

These phosphorylated residues act as docking sites for a number of other signaling intermediates via their SH2 homology domains.\textsuperscript{70,78}

Although a number of signaling pathways are activated by Epo stimulation, perhaps the most extensively studied pathway involves the Signal Transducer
and Activator of Transcription (STAT)5. Jak2 phosphorylates STAT5 which initiates dimerization of the protein and translocation to the nucleus where it binds DNA and initiates the transcription of anti-apoptotic genes such as Bcl-X, a Bcl-2 family member protein. STAT5 deficient mouse embryos are severely anemic. In addition to STAT5, activation of GATA-1 transcription factor is necessary for erythroid cell development based on studies of null mouse models. GATA-1 regulates a number of genes involved in erythroid differentiation including EpoR and Sp1 genes as well as inhibiting apoptosis in erythroid precursors. GATA-1 was shown to be activated through the phosphotidyl-3 kinase (PI3K) /Akt pathway. Phosphorylation of GATA-1 by PI3K is necessary for Epo induced growth of erythroid progenitors. The PI3K pathway is activated in part through receptor binding of Grb2 and Vav. Vav and Grb2 additionally activate Ras and Rac, members of the Rho GTPase family of proteins. The GTPases are molecular switches that cycle between active GTP-bound and inactive GDP-bound states and are involved in the regulation of cellular component trafficking and cytoskeletal changes. GTPases will be discussed in more detail later. Lyn kinase is also activated and associated with the receptor at the cell surface. Lyn kinase phosphorylates both the EpoR and Stat5 to cooperate in the potentiation of Epo signaling. The mitogen-activated protein kinase (MAPK) pathway is similarly activated after Epo stimulation, and has been shown to be additionally responsible for the activation of Rho kinase. Regardless of the pathway involved, EpoR signaling induced by cytokine stimulation is essential for not only basal RBC maintenance
and production, but also in anemia caused by erythropoietic insult or hypoxia. Therefore aberrations of this pathway are critical limiting factors in the pathogenesis of anemia in select hematologic disease pathogeneses, and provide possible therapeutic targets.

**Negative regulation of EpoR signaling.** Negative regulation of the EpoR pathway is primarily coordinated through negative feedback loops. This regulation is accomplished by a number of phosphatases and a family of proteins known as the suppressors of cytokine signaling (SOCS). The SOCS proteins bind Jak2 inactivating it, block STAT5 binding sites on the receptor, and ubiquitinates signaling proteins, all of which halt Epo induced signaling. The major phosphatases involved in the negative regulation of this pathway include hematopoietic cell phosphatase (HCP, SHP-1 or PTP1C) and CD45. SHP-1 dephosphorylates Jak2 causing its inactivation and halting downstream signaling. The transmembrane phosphatase, CD45, is essential for both T-cell and B-cell regulation, as well as EpoR signaling. CD45 dephosphorylates a number of proteins including Jak2. Lastly, receptor recycling and turnover also regulates Epo induced signaling. After ligand binds the receptor, it is immediately ubiquitinated and either degraded or recycled back into the cell.

**EpoR signaling in MDS.** Patients with MDS have comparable levels of both endogenous Epo and normal cellular membrane density of EpoR, however, clear functional inadequacies are evident compared to normal controls. Given
that there is no identified loss of ligand binding to the receptor, impaired activation of STAT5 must relate to a deficiency in the signaling pathway.\textsuperscript{49} Unfortunately, the exact mechanism underlying signal impairment remains elusive. Hoesflood et al. reported that there is a marked decrease in Epo induced DNA transcription and binding of \textit{GATA-1} in MDS patients compared to normal controls.\textsuperscript{49} A loss of GATA-1 transcriptional activity was followed by decreased maturation in response to Epo, and loss of erythroid or Epo responsive cells.\textsuperscript{49} It was also found that STAT5 activation after Epo stimulation was either undetectable or at very low levels in MDS progenitors.\textsuperscript{49} The diminished STAT5 activation coincides with a decrease in erythroid colony-forming capacity and Epo induced DNA synthesis.\textsuperscript{49} Furthermore, the decrease in STAT5 activation was not due to low numbers of erythroid cells, suggesting that the site of dysfunction is an early event likely preceding activation of STAT5.\textsuperscript{49} Furthermore, it suggests that ineffective erythropoiesis in MDS may be caused by a defect in the signaling cascade. Since Hoesflood et al. published their reports in 1997, the mechanism of decreased signaling has still yet to be identified. In this manuscript, we propose that impairment in lipid raft signaling platforms are responsible for decreased STAT5 activation and provide a novel finding on the disease biology of MDS and possible therapeutic strategies.

\textbf{Lipid Rafts}

\textit{Identification and composition.} The plasma membrane is a fluid
phospholipid bilayer. Within this fluid membrane structure, discrete entities composed of tightly packed sphingolipids and cholesterol ‘float’ freely. These platforms are termed lipid, or membrane, rafts.\textsuperscript{99,100} The rigidity and relatively ordered state of lipid rafts are a consequence of the saturated acyl chains on the sphingolipids in contrast to the unsaturated fatty acyl chains of the phospholipids.\textsuperscript{101} High levels of sphingolipids and cholesterol in the apical domain of polarized epithelial cells was the first evidence of the existence of lipid rafts.\textsuperscript{100,102-104} Since these initial findings almost 20 years ago, accumulating evidence reveals that rafts are formed through a lateral association of sphingolipids that are held together by their hydrocarbon chains and tightly associated cholesterol molecules.\textsuperscript{102,105} Rafts are associated with glycosylphosphatidylinositol (GPI)-anchored proteins, cytokine receptors, kinases, and GTPases that mediate intracellular signaling in response to varied external stimuli (Figure 6).\textsuperscript{102,105} The sterol composition of the lipid rafts renders them relatively insoluble to detergent lysis at 4°C and therefore they are also sometimes referred to as detergent resistant membranes (DRM) or detergent-insoluble, glycolipid-enriched complexes (DIGs).\textsuperscript{102,106} There is reported to be 10x more cholesterol in the raft fractions compared to non-raft fractions, however, how much of the plasma membrane is actually accounted for by lipid rafts remains controversial.\textsuperscript{106} Studies of raft size vary greatly but reports suggest that they may range anywhere from 10-200nm.\textsuperscript{101,102} Although exact sizes of rafts are disputed, it is agreed that they are relatively small under steady state conditions, but that they increase in size due to aggregation upon external stimuli,
Figure 6. Lipid Rafts. Lipid rafts are composed of tightly packed sphingolipids and cholesterol forming platforms that contain GPI anchored proteins, cytokine receptors, kinases, and GTPases that mediate cell signaling, a process which is necessary to mediate downstream signaling pathways.\textsuperscript{102,105}

**Raft formation.** The process of raft assembly and aggregation is accomplished through intricate coordination with the actin cytoskeleton.\textsuperscript{101} The actin cytoskeleton is a dynamic assembly of subunits that changes in response to external stimulation.\textsuperscript{107} Interaction between the actin cytoskeleton and lipid rafts is suspected to be involved in protein trafficking as well as translocation of raft subunits from the Golgi to the plasma membrane.\textsuperscript{101} Cholesterol is produced in the ER, however, sphingolipids are produced in the Golgi.\textsuperscript{102} Raft assembly is
initiated in the ER, but after component glycosylation, moves to the Golgi where
manufacturing occurs before translocation to the membrane.\textsuperscript{102,107,108}
Sphingolipid and cholesterol stores are maintained at the trans Golgi network
and are available under appropriate conditions to promote rapid coalescence and
recruitment of signaling molecules.\textsuperscript{107} Each of these processes is mediated
through the actin cytoskeleton.\textsuperscript{102,107,108} Although actin itself does not interact
with the lipid rafts, these proteins act as a scaffolding between the raft
constituents and cytoplasmic proteins, and also serves as tracks to guide protein
transport.\textsuperscript{101} The aggregation of rafts is dependent upon reorganization of
filamentous (F)-actin.\textsuperscript{101,107} These rearrangements are coordinated in part
through the dedicator of cytokinesis 2 (DOCK2) and Rac GTPases.\textsuperscript{101} RhoA and
Rho associated-coil-coil-containing protein kinase (ROCK) are also particularly
important for this process.\textsuperscript{101} Rac GTPases will be discussed in greater detail
shortly. The actin cytoskeleton is not only dynamic, but bidirectional, therefore
the same pathways leading to the formation of the rafts and shuttling to the
membrane, are also used in raft recycling and negative feedback loops.\textsuperscript{107}
Endocytosis of rafts may lead to recycling back to the plasma membrane,
recycling back to the Golgi, or dissociation.\textsuperscript{109}

\textbf{Functions.} Since their identification in polarized epithelial cells, lipid rafts
have since been implicated in a number of cellular processes. For example, rafts
are the point of entry for a number of infectious pathogens. Some pathogens
known to be dependent on rafts for cell invasion include the malaria parasite,
*Plasmodium falciparum*, the influenza virus, and HIV-1.\(^{102,105,110-112}\) Disruption of lipid rafts inhibited entry of each of these pathogens indicating a critical role of the rafts in cell penetration and infection.\(^{102,105,110-112}\) Other raft functions can be exemplified by showing the diversity of proteins that are functionally dependent on membrane rafts. Both the B-cell receptor (BCR) with CD20 and CD19/CD21 complexes, and the T-cell receptor (TCR) are present in raft fractions.\(^{101,113}\) The dependence of T-cell activation on lipid rafts has been extensively studied and will be discussed in the next section. There are also a number of cytokine receptors known to reside in rafts, including the vascular endothelial growth factor (VEGF), granulocyte-macrophage colony stimulating factor (GM-CSF), epidermal growth factor (EGFR), and insulin-like growth factor receptor (IGFR).\(^{114}\) The presence of these cytokine receptors (and others) in lipid rafts signifies the importance of rafts in cytokine signaling. The FcεRI receptor, important for IgE signaling resides in lipid rafts suggesting a role in allergic reactions as well as parasite immunity.\(^{113}\) The death receptor complex, Fas/CD95, also resides in lipid rafts implicating a role of in apoptosis.\(^{115}\) The ATP-binding cassette transporters (ATP transporters) including P-glycoprotein (Pgp) and Multidrug resistance-associated protein 1 (MRP-1) are located in rafts, suggesting a role in multidrug resistance.\(^{116}\) G-protein coupled receptors (GPCRs), such as the dopamine receptors, are constitutively associated with lipid rafts implicating an importance for neuronal signaling.\(^{116-119}\) Ferroportin, the protein responsible for iron export in macrophages, is dependent on lipid rafts.\(^{120}\) Integrins involved in cell to cell contact, migration, and metastasis also reside in
the rafts. Rafts are also important for endocytosis, a process by which receptors are internalized into the cell after ligation. Some of the most highly raft associated proteins are the Src family kinases, such as Lyn kinase. Lyn kinase acts as a mediator between raft domains and signaling effectors further supporting the important role of rafts in cell signaling. The presence and functional dependence of these proteins on rafts localization demonstrates the diversity and magnitude of cellular processes associated with these membrane microdomains.

**T-cell signaling.** The role of lipid rafts in signaling has been most extensively studied in T-cell activation. Upon stimulation, T-cell receptors (TCR) coalesce in the lipid rafts, compartmentalizing with associated signaling proteins. TCR is associated with GPI-anchored proteins and the clustering of lipid rafts marks localization of the immune synapse. This clustering occurs via the raft constituent, ezrin, an ERM (ezrin, radixin, moesin) protein that links the plasma membrane and the actin scaffolding by crosslinking the actin filaments. After TCR clustering, Zap-70 is recruited to the raft to act as a bridge between the TCR and downstream signaling molecules. Coincidentally, Zap70 recruitment to rafts is also necessary for the activation and recruitment of protein kinase C-βII (PKC-βII) in BCR signaling. Several other T-cell intermediates are then recruited to the raft fractions after TCR activation including CD3ζ chain, SLP76, PKCθ, PLCγ1. Both PI3K and CARMA1 (caspase recruitment domain membrane-associated guanylate kinase 1) are also
recruited to rafts in both T- and B-cells. Furthermore, the signal attenuating tyrosine phosphatase, and transmembrane protein, CD45, is sequestered out of the raft membrane upon TCR activation. Raft disruption blocked the aggregation of TCR and the recruitment of these intermediates ultimately inhibiting T-cell activation. The coordination of positive effector recruitment and negative regulator dismissal through raft organization is necessary to optimize T-cell signaling.

**Immune synapse.** The immune synapse is the site of cell to cell contact for both T- and NK cells, and has abundant raft aggregation, allowing optimal spatial organization of receptors and signaling intermediates. Raft aggregation promotes the reorganization of the cytoskeleton that is necessary for the formation of the immunological synapse. Raft aggregation and intermediate recruitment at the synapse is ultimately responsible for the ability of both the T- and NK cells to perform their immunological functions. Actin filament assembly and immune synapse formation in activated T-cells is dependent on the Arp2/3 complex, Wiscott-Aldrich syndrome protein (WASp), the GTPase effector, mammalian homolog of diaphanous formin, mDia1, and the Rho/ROCK/LIMK pathway. Disruption of these pathways result in impaired lipid raft formation and limited the ability to form immunological synapses. After receptor engagement at the immune synapse, rafts are endocytosed, following integrin mediated detachment from the substratum, which is also dependent on actin polymerization regulated in part by another GTPase Arf6. These
GTPase activated cytoskeletal rearrangements will be discussed further under the *GTPases* section of this manuscript. Raft disruption significantly and negatively impacts immune synapse fidelity and decreases TCR activity, illustrating the importance of rafts in immune response. It is now well accepted that lipid rafts serve as signaling platforms that sequester receptors and downstream signaling effectors sequester to facilitate signal transduction for a number of receptor pathways, and in diverse cell types.\(^{102}\)

**Raft mediation of signaling cascades.** Currently there are a number of receptor pathways known to be initiated or mediated by rafts, however, the question arises, how does this occur? Currently, there are several suggested theories to explain how rafts coordinate receptor signaling, or vice versa, how signaling cascades initiate raft aggregation. First, the affinity of cholesterol and signaling intermediates may increase after raft coalescence.\(^ {113}\) Alternatively, rafts may be induced to coalescence upon protein aggregation at the plasma membrane.\(^ {113}\) Others suggest that raft activated enzymes may produce water soluble phosphor-oligosaccharides that may activate downstream molecules, or, that activation and/or aggregation of rafts induces the clustering of GPI-anchored proteins, and that these proteins then in turn activate downstream signaling molecules.\(^ {102,105}\) Regardless of how it is accomplished, it is clear that raft activation is important for signal initiation, and that rafts themselves are activated and aggregated upon receptor-ligand engagement.


**Raft Experimentation.** There have been a number of tools used to visualize lipid rafts including electron microscopy, fluorescent resonance energy transfer (FRET), single particle tracking, immunofluorescence, photonic force microscopy, chemical crosslinking, and ultra-centrifugation in sucrose density gradients. The composition of sterols in the lipid raft domains make them relatively detergent-insoluble, particularly to Triton-X, at 4°C which allows for their isolation and identification. Upon ultracentrifugation in sucrose density gradients, the insoluble raft fractions will float to lower density matrices allowing for study of both the rafts and their associated proteins. Another useful tool in the study of lipid rafts takes advantage of the constituent raft ganglioside, GM-1. The endotoxin, cholera toxin, from *Vibrio cholera*, is comprised of subunits A and B (CT-B), and CT-B has high affinity for GM-1. This selective interaction facilitates identification of lipid rafts in a number of assays. CT-B may be conjugated to fluorochromes for GM-1 detection via immunofluorescence and flow cytometry, or to horseradish peroxidase for western blot detection. Many of these methods will be described in further detail in Chapters 2 and 3 of this manuscript. Another important feature of lipid rafts is the ability to disrupt them with the use of cyclic oligosaccharadides, i.e. cyclodextrins. Cyclodextrins bind to and extract cholesterol from the membrane effectively disrupting raft integrity with accompanying loss of all associated proteins. Lipid raft integrity is highly dependent on cholesterol and therefore its removal is of bitter consequence to raft fractions. Beta-cyclodextrins are the most highly attracted to cholesterol and therefore are most
effective at removing the cholesterol from the plasma membrane.\textsuperscript{133} Furthermore, the solubility of β-cyclodextrins increases by adding methyl groups, and methyl-β cyclodextrin (MβCD) is the most commonly used agent for raft disruption.\textsuperscript{106,133} Cholesterol intercalating agents, such as nystatin, are alternative although less effective disrupting agents.\textsuperscript{109,113} Instead of removing cholesterol from the membrane as is the case with MβCD, nystatin binds to cholesterol in the membrane, sequestering it and causing disruption of raft dependent signaling cascades.\textsuperscript{106,134} Nystatin is an anti-fungal with structure similarity to amphotericin B, as opposed to an HMG-CoA reductase inhibitor statins that block cholesterol synthesis.\textsuperscript{135}

As mentioned above, GTPases are associated with the raft fractions and are important to drive reorganization of the cytoskeleton and as such warrant further discussion.

**GTPases**

**Ras superfamily.** The Ras superfamily of GTPases is comprised of 154 members, grouped into 5 major branches based on sequence similarity, and are involved in a number of cellular processes including signal transduction, cell cycle regulation, and cytoskeletal reorganization (Figure 7).\textsuperscript{136} Furthermore, Ras GTPases are associated with the lipids in the plasma membrane via isoprenylation which is necessary for membrane attachment.\textsuperscript{137}
Figure 7. Ras superfamily. The Ras GTPase superfamily is comprised of 154 members that can be grouped into 5 major branches based on sequence similarity with 9 additional members. Some of the cell process regulated by each family is provided.

The Ras GTPases are small proteins which shuttle between inactive GDP-bound states, and active GTP-bound states.\textsuperscript{136} The transition between GDP-bound and GTP-bound is accomplished through guanine nucleotide exchange factors (GEFs).\textsuperscript{136} Hydrolysis of GTP back to GDP is accomplished through GTPase-activating proteins (GAPs).\textsuperscript{136} Rho and Rab GTPases are additionally regulated through guanine nucleotide dissociation inhibitors (GDI) which can block binding of GEFs and GAPs, and prevent the association of the GTPases to the plasma membrane (Figure 8).\textsuperscript{136,138} Gene mutations of proteins that cause an upregulation of Ras can be found in about 33% of all human cancers, however, mutations of Ras itself are often not observed.\textsuperscript{136} Most commonly
Figure 8. GTPase regulation. GTPases switch from inactive GDP bound states and active GTP bound states via guanine exchange factors (GEFs) and GTPase activating proteins (GAPs). Rho and Rab GTPases are also regulated by guanine dissociation inhibitors (GDI) which prevent GEF and GAP binding to the GTPases, and can block association with the plasma membrane.

these activating mutations are found in GEFs and GAPs. One of the most important functions of the Ras GTPases is reorganization of the actin cytoskeleton.

**Cytoskeleton.** The actin cytoskeleton performs a number of vital cell functions including spatially organizing cellular components, serving as the messenger between the intra- and extra-cellular environments, regulating cell
movement and shape, and regulating cargo transport throughout the cell. The cytoskeleton continually accommodates cellular changes based on stimuli through reorganization of actin monomers. Actin polymerizes to form filaments which can be bundled or branch out to form dynamic networks. The cytoskeleton has three major components; microtubules, actin filaments, and intermediate filaments. Microtubules are the stiffest and most complex components and are best described in their role in cell division. Microtubules are unique in the regard that they are either in a state of polymerization or depolymerization, but never both simultaneously. Interestingly, microtubule formation is activated by the GTPase Rac1 which in turn activates the polymerization of microtubules creating a positive feedback loop. Actin filaments are also highly organized, however, are less rigid than microtubules. Actin filaments form a scaffold that is held together by spectrin, forming a hexagonal lattice that lies beneath the plasma membrane and allows tethering of membrane associated proteins including GTPases. Actin filaments are important for cytoskeletal reorganization based on external stimuli and are key to lipid raft aggregation in response to these stimuli. Furthermore, actin filaments are involved in the trafficking to and from the plasma membrane, as well as in the internalization of ligand bound cytokine receptors. These processes are all regulated by Rho GTPases. Both epidermal growth factor (EGF) and platelet-derived growth factor receptor-β (PDGFR-β) are known to be endocytosed through actin reorganization and are dependent on the activity of GTPases. Unlike microtubules, actin filaments are undergoing simultaneous polymerization
and depolymerization, allowing constant rearrangement based on environmental stimuli. Actin filaments continue to polymerize so long as there are available monomers suggesting regulation by associated factors such as GTPases. Intermediate filaments are the least rigid components of the cytoskeleton, however, they are the most efficient at resisting tensile forces making them particularly important for actively circulating cells. One of the most well studied branches of the Ras family are the Rho GTPases, which in addition to a number of other cellular functions, are heavily involved in regulating these actin cytoskeleton formations.

**Rho GTPases.** Two of the most highly studied actin cytoskeletal structures are stress fibers and membrane ruffles. In 1992, Ridley, Hall, and colleagues found that the formation of these cytoskeletal elements was induced by the activation of the Rho GTPases. This observation was the first suggesting GTPases are involved in regulating cytoskeletal reorganization. The Rho GTPases are comprised of 20 members that can be separated into 8 families based on sequence similarities (Figure 9). The three branches of the Rho family known to be involved in action skeleton organization include the Rho-like family (RhoA, RhoB, and RhoC), Rac-like family (Rac1, Rac2, and Rac3), and Cdc42-like, whereas the best studied are RhoA, Rac1, and Cdc42. Although there is a high degree of sequence similarity between these proteins, they have both overlapping and distinct functions. Additionally, there is substantial crosstalk between them. Cytokine signaling induces membrane
Figure 9. Rho GTPase family. The Rho GTPase family is comprised of 20 members that can be grouped into 8 subcategories based on sequence similarity. The Rac-like, Cdc42-like, and Rho-like families are involved in signal transduction and actin cytoskeleton reorganization.

ruffle formation at the leading edge of migrating cells and is controlled by Rho GTPases. Activation of the actin cytoskeleton by Rho family members is critical to a number of cellular processes including cell polarity, cell motility, endocytosis, cell cycle regulation, vesicle trafficking, stress fiber formation, and focal adhesions. Cytoskeletal reorganization via active Rho GTPases has been implicated in cell metastasis and therefore may have a role in disease progression. Receptor tyrosine kinase (RTK) phosphorylation prefers proximity to lipid rafts, and reorganization of the cytoskeleton induces raft aggregation and the recruitment of these receptors, processes that are
dependent on the GTPases. Upon cytokine stimulation, Rho activates lipid raft aggregation through the Rho kinases, ROCKI and ROCKII (ROCK). ROCK phosphorylates LIM kinase (LIMK) which then inactivates cofilin through inhibitory phosphorylation. When cofilin is phosphorylated it is no longer able to bind to actin, and thereby, is unable to depolymerize actin filaments, thus inducing actin polymerization. Rho subfamily GTPases also stimulate actin polymerization and stress fiber formation via direct phosphorylation and activation of the myosin light chain (MLC) and through ROCK which additionally functions to inhibit MLC phosphatase. ROCK is also important for the migration of macrophages and neutrophils which is accomplished through regulation of PTEN. Additionally, Rho acts through another GTPase effector, mDia. mDia is part of the formin family of proteins that are known regulators of the actin cytoskeletal. Active mDia promotes actin polymerization by promoting binding of monomers to branched ends of filaments and is important for the interactions of endosomes and the cytoskeleton. Interestingly, the gene encoding mDia1, DIAPH1, is located within the commonly deleted region of del(5q) and mice lacking DIAPH1 develop a myelodysplastic phenotype implicating a role in disease pathogenesis. In del(5q) MDS, it is thought that loss of DIAPH1 inhibits the sensing ability of the actin cytoskeleton and somehow promotes the expansion of the del(5q) clones although it is not clear how this is done. Lastly, Rho actives actin polymerization through WASp and Arp2/3 proteins. When activated, Rho proteins activate downstream signaling effectors and actin skeleton reorganization.
Rac GTPases. The Rac family GTPases are involved in lamellipodia, membrane ruffle formation, axon growth, cell adhesion, phagocytosis, and cell differentiation.\textsuperscript{140,143} Rac proteins very actively induce actin polymerization through the WASp family protein, WAVE/Scar (Figure 10).\textsuperscript{143} WAVE/Scar activates the Arp2/3 proteins which are responsible for the binding of actin monomers to filaments, causing the branching and weblike matrix of the actin cytoskeleton.\textsuperscript{143} Rac activates WAVE through 2 mechanisms; 1) the activation of IRSp53, and 2) the forced dissociation of Nap125, PIR121, and/or HSPC3000 from WAVE/Scar allowing the latter to activate the Arp2/3 proteins.\textsuperscript{140,143} Rac also inhibits the actin depolymerizing protein, coflin, further inducing actin polymerization.\textsuperscript{140} Rac proteins are responsible for lamellipodium and membrane ruffling and extension.\textsuperscript{140} These likely occur through activation of the DOCK proteins.\textsuperscript{140} Although the Rac proteins have very similar sequences, they seem to have distinct roles depending on cell type.\textsuperscript{140} Rac1 is observed in most cell types whereas Rac2 is predominantly expressed in hematopoietic lineages, and Rac3 is found mainly in the brain suggesting specific roles of Rac members in tissue specific contexts.\textsuperscript{140}

Cdc42. Cdc42 induces filopodia formation and is involved in cell polarization and protrusion. Additionally, Cdc42 (and Rac1) are involved in cell cycle regulation particularly at the G1/S checkpoint, through a MAPK dependent pathway.\textsuperscript{146,147}
Figure 10. Rho GTPase activation of cytoskeletal reorganization. The Rho GTPases regulate actin cytoskeletal reorganization through a number of different pathways shown here.

The filopodia formed by Cdc42 are composed of bundles of F-actin and serve as "scouts" sensing the cell surroundings and relaying messages to the inside of the cell. The downstream targets of Cdc42 are WASp (that activates the Arp2/3 proteins), and mDia, which induces formation of unbranched actin filaments. In summary the Rho GTPases induce actin polymerization and cytoskeletal reorganization through a number of mechanisms including activation of actin nucleating Arp2/3 proteins, increasing myosin phosphorylation, through ROCK which inhibits MLC phosphatase to augment LIMK phosphorylation, and through the formin proteins such as mDia (Figure 10).
**GTPases and hematopoiesis.** The Rho GTPases have key functions in hematopoiesis. Some of the first evidence for this derives from investigations showing that HSCs deficient for Rac1 and Rac2 have decreased myelo-and erythropoiesis. Subsequent studies showed that Rho GTPases regulate HSC survival, proliferation, and engraftment in transplantation models. The Rho GTPases also regulate HSC interaction with the bone marrow niche. Early erythropoiesis in the bone marrow is dependent on Rac1 and Rac2, which are responsible for cytoskeletal arrangements in erythrocytes. The GTPases function in erythrocytes via activation of mDia2. Furthermore, mDia2 was found to be necessary for the maintenance of myeloid homeostasis, while RhoB deficiency induces myelodysplasia in mice. Rac was also found to be important for the creation of the actin scaffolding at the plasma membrane in erythrocytes. Furthermore, Rac GTPases are necessary for erythrocyte enucleation via mDia2. In their review, Mulloy et al. note that GTPases are involved in nearly every step of hematopoiesis and differentiation of all lineages.

**Rho GTPases and IMiDs.** Rho GTPases are activated by IMiDs resulting in cytoskeletal reorganization. IMiD activated RhoA and Rac1 induced cytoskeletal reorganization in lymphocytes within minutes, whereas Cdc42 did not have similar effects even after extended periods of exposure. Pomalidomide and LEN induced F-actin polymerization was dependent on RhoA and Rac1 activity. Pomalidomide increased microtubule stabilization and actin
polymerization at the apex of migrating cells through ROCK1. These effects were specific to cell lineages as they were not found in all cell lines tested. However, these reports were the first to indicate that activation of GTPases is a biological effect of IMiD treatment. Although it is now known that the IMiDs activate the GTPases, it is not clear how this activation is mediated. We hypothesize here, that actin reorganization via activation of the GTPases after IMiD treatment may occur through inhibition of GTPase E3 ubiquitination.

**E3 Ubiquitin ligases**

**Overview.** Ubiquitination is a process by which ubiquitin (Ub), a 76 amino acid peptide, is transferred to a lysine residue on a target protein marking that protein for a number of cellular processes including intracellular trafficking, gene regulation, DNA repair, and proteasomal degradation. Ubiquitin has 7 lysine residues that may be used for a number of different ubiquitin chain combinations. Ubiquitin can be added linearly or may branch out forming complex structures, the functions of which are not completely understood (Figure 11). The complex nature, and vast possibilities of Ub chains, may be considered as an “ubiquitin code,” which may relay specific signals to target proteins directing a number of different outputs. Polyubiquitination (occurring at lysine 48) of at least four subunits, in general, targets a protein for degradation, whereas, mono-, di-, or tri-ubiquitination (on lysine 63) often signals other cellular tasks. Polyubiquitination causes degradation by targeting the protein to the
Figure 11. The ubiquitin code. The process of substrate ubiquitination targets a substrate for a number of cellular processes including gene regulation, DNA repair, and proteasomal degradation. Ubiquitin monomers may be added linearly, or may form branched chains. The sequence and structure of these chains marks the substrate for different functions although the exact code is not completely understood.

26S proteasome. The 26S proteasome is comprised of 2 subunits, the 20S proteolytic core and the 19S regulatory unit capping the 20S at both ends. When a ubiquitinated protein is delivered to the proteasome it is de-ubiquitinated then unfolded by ATPases at the 19S subunit. It is then delivered to the core proteolytic subunit where degradation occurs. Ubiquitinating complexes are composed of three subunits; the ubiquitin-activating enzyme, E1, which uses ATP to transfer a ubiquitin molecule to the conjugating enzyme, E2, which acts as the donor for the ubiquitin ligase enzyme, E3 that is responsible for the transfer of the ubiquitin from the E2 to the substrate receptor. There are two main classes of E3 ligases, HECT (homologous to E6-AP C-terminus) and RING (really interesting new gene) domain ligases which are presumed to have different target motifs. Currently, there are only about 30
known HECT ligases but more than 600 suspected RING domain ligases. Interestingly, there are only two known E1s and less than 40 E2s suggesting the E3s are responsible for specificity. The major difference between RING and HECT E3 ligases, in addition to domain and structure differences, is how they transfer the ubiquitin group from the E2 to the substrate receptor (Figure 12). RING ligases directly transfer the ubiquitin from the E2 to the acceptor acting as a platform connecting the E2 and substrate. However, HECT ligases first transfer the ubiquitin to an activated cysteine residue on the E3 ligase before then transferring it to the substrate. Recently, pseudo RING/HECT hybrids were identified, that are collectively referred to as RING-in-between-RING (RBR) ligases and will not be discussed in this manuscript.

![Figure 12. E3 ligases.](image)

**E3 ligase regulation.** Regulation of E3 ligases is often accomplished by post-translational modification and/or substrate availability. Both the E2 and E3 subunits may be phosphorylated which is one mechanism by which regulation is achieved. E3s can also be regulated by specific binding partners, by
ubiquitin like peptides, or by small molecules changing the affinity of the E3 for either the E2 or target Ub acceptor. Additionally, there are about 100 deubiquitinating enzymes (DUB) that regulate E3 ligase activity by removing ubiquitin groups. Of particular importance is the fact that E3 ligases themselves may be ubiquitinated and targeted for degradation either by other E3 ligases, or in many cases, by auto-ubiquitination. Since E3 ligases effect so many cellular processes, their regulation is of utmost importance. As such, deregulation of E3 ligases have been linked to a number of human disorders including Parkinson’s disease and many different malignancies.

Plasma membrane development and protein turnover. The translocation of plasma membrane machinery and associated proteins are controlled by the secretory pathway (which brings components to the cell surface) and the endocytic pathway (which is responsible for the internalization or recycling of membrane components to the intracellular compartment) both of which are controlled in part through ubiquitination complexes. Although ubiquitination does not seem to play a role in the transport of proteins from the ER to the Golgi, it is responsible for the degradation of misfolded ER proteins, and plays a role in Golgi sorting. Any misfolded proteins that escape ER degradation, may be marked for degradation by what is known as the Golgi complex quality control (GQC) system. E3 ligases are also responsible for protein transport to the plasma membrane through vesicles, although the exact mechanisms are not completely understood. There are numerous reports
suggesting dependence of endocytosis on ubiquitination, particularly of plasma membrane components.\textsuperscript{162} Both RTKs and GPCRs are endocytosed after ligation through endocytic pathways which are dependent on ubiquitination.\textsuperscript{162} Upon stimulation these receptors are ubiquitinated triggering their internalization.\textsuperscript{162} The specific roles and specific ligases in endocytosis are not fully understood in part due to the number of roles the endocytic pathway plays.\textsuperscript{162} For example, receptor internalization may direct the receptor to be either degraded by the proteasome or recycled back to the plasma membrane.\textsuperscript{162} In fact, proteins may be sent back to the plasma membrane, sent to the proteasome, or sent to the Golgi for recycling.\textsuperscript{162} Also, not all endocytosed proteins are ubiquitinated.\textsuperscript{162} It is believed that endocytic sorting may involve the cooperation between E3 ligases and DUBs, a process that is complicated by the fact that internalized plasma membrane proteins can interact with other cytoplasmic proteins to promote the formation of new complexes with new cellular roles for the recycled plasma membrane component, the E3 ligases involved in its internalization, and cooperating effects of the DUBs.\textsuperscript{162} It is believed that when the E3 ligase is associated with ESCRT (endosomal sorting complex required for transport), the substrate protein will be bound to the lysosome and ultimately for degradation. However, further studies are necessary to confirm these findings.\textsuperscript{162}

The role of E3 ligases is pivotal to a number of cellular processes including regulating components of the plasma membrane and the turnover of important signaling receptors. Although there are hundreds of ubiquitin ligase
complexes, it is important for purposes of this manuscript to further discuss three specific RING family members, cereblon, MDM2, and RNF41.

**Cereblon.** Cereblon (CRBN) is a 442 amino acid protein that is part of an E3 ubiquitin ligase complex that includes Cullin 4, regulator of cullins-1 (Roc1), and damaged DNA binding protein-1 (DDB1). Roc1 and DDB1 form the catalytic core of the complex that interacts with E2 enzymes. DDB1 also connects Cul4 with the substrate. The exact role of CRBN in the complex is unknown. CRBN is highly expressed in the human brain and may have a role in memory and learning. Truncations of CRBN are associated with mental retardation. Although the importance of CRBN in brain development is not new, studies in the last 3 years have shown an important role in IMiD activity. Ito et al. reported that the direct binding target of thalidomide responsible for its teratogenic effects, is the CRBN-DDB1 complex. The Cul4-E3 ligases are known to be important for embryonic development and after more than 40 years of investigation, and over 30 different hypotheses, direct binding of thalidomide to CRBN is now accepted as the mechanism by which thalidomide induces teratogenicity. However, there are likely other factors that contribute to teratogenicity since malformations are observed in specific tissues (brain and limbs) despite the fact that CRBN is expressed in all cell lineages. Interestingly, thalidomide is not teratogenic in mice despite 95% sequence similarity between human and mouse CRBN, and similar binding affinities of thalidomide and CRBN. There are a number of hypotheses proposed to explain this including
differential breakdown of hydrolyzed byproducts of thalidomide between mice and humans, differences in the biological activity of thalidomide, and a lack of ROS formation induced by thalidomide in mice due to a stronger anti-oxidant response.\textsuperscript{43}

Direct binding of thalidomide to CRBN causes inhibition of the ubiquitin ligase function of the CRBN complex.\textsuperscript{43,169} In addition to the inhibition of ligase activity on substrates, IMiDs also inhibit the auto-ubiquitination ability of CRBN further regulating its function.\textsuperscript{39,168} Recent investigations have implicated CRBN in the anti-neoplastic effects of thalidomide and LEN in MM.\textsuperscript{168,170,171} This finding implies that the E3 ligase activity of CRBN is necessary for degradation of some regulators important in the maintenance and/or survival of MM cells.\textsuperscript{168,170,171} These findings were not specific to thalidomide as both pomalidomide and LEN were also found to bind to CRBN, and this interaction was equally as important in the anti-proliferative effects in MM cells.\textsuperscript{170} Not surprisingly, pomalidomide or LEN resistant myeloma cells have decreased levels of CRBN.\textsuperscript{170} The importance of these findings is threefold; first, they are the first time a direct binding partner of the IMiDs has been identified. Second, the importance of CRBN in mediating varied biological effects of the IMiDs in MM may increase knowledge of MM disease biology as well as other hematological malignancies responsive to IMiDs such as MDS. And lastly, the finding that IMiDs can inhibit E3 ligase activity has major implications in what is known about the overall biological effects of the IMiDs.
**MDM2.** The murine double minute-2 (MDM2) protein is the major negative regulator of p53.\(^{172}\) P53 is well known to be involved in a number of cellular processes including cell cycle regulation, apoptosis, DNA repair, and senescence.\(^{173}\) MDM2 is capable of negatively regulating p53 by two mechanisms.\(^{172,173}\) First, MDM2 binds p53 at the N-terminus of MDM2, thereby preventing p53 binding to DNA and preventing transcriptional activity.\(^{172,173}\) Although, MDM2 blocks p53 binding to DNA, its primary regulation is through ubiquitination and ultimately degradation by the proteasome.\(^{172,173}\) Since the RING domain is in the C-terminus of MDM2, the two methods by which MDM2 regulates p53 can be ascertained after a stimulus is provided.\(^{172,173}\) Regulation of p53 is of utmost importance as it is upregulated under cytotoxic stress (such as genotoxic stress, hypoxia, heat shock or others.)\(^{173}\) However, high levels or sustained activation of p53 promotes apoptosis, indicating the need for strict regulation and negative feedback.\(^{172,173}\) Interestingly, p53 activates MDM2 transcription creating its own negative feedback loop.\(^{172}\) When p53 is activated by stress it activates MDM2 transcription, MDM2 will then bind to p53 targeting it for degradation thereby completing the loop.\(^{172,173}\) In addition to p53, MDM2 binds and ubiquitinates several other proteins with p53 sequence homology including p73, p63, p51, and E2F1 blocking the transactivation of each.\(^{172,173}\) Although there are a number of E3 ligases that ubiquitinate p53 including the HECT E3 ligase, E6AP, Cul4-DDB1 complex, CBP/p300 and others, MDM2 is thought to be the primary regulator.\(^{173}\) MDM2 gene overexpression is observed in up to 7% of all cancers, with up to 20% of all soft tissue tumors.\(^{172}\) Therefore,
MDM2 is a potential therapeutic target for functional interference to stabilize p53 and induce apoptosis of malignant clones.\textsuperscript{172} Additionally, the MDM2 homolog, MDMX, also ubiquitinates p53 in both an MDM2 dependent and independent manner. MDM2 and MDMX can heterodimerize further regulating their activity in p53 degradation.\textsuperscript{172-174} Both MDM2 and MDMX deficient mouse embryos are non-viable.\textsuperscript{172,173} Interestingly, when p53 was knocked out in MDM2 or MDMX embryos, viability of the embryos was restored indicating that the loss of MDM2 or MDMX caused cell death in a p53 dependent manner.\textsuperscript{172-174}

Our laboratory and colleagues recently reported that LEN inhibits the auto-ubiquitination of MDM2, stabilizing the protein, and promoting the degradation of p53.\textsuperscript{175} In del(5q) MDS, there is accumulation of p53 resulting from nucleolar stress and the release of unbound ribosomal proteins in the nucleus. This results from haplosufficiency of \textit{RPS14}.\textsuperscript{23} The unbound ribosomal proteins bind to and promote the degradation of MDM2 causing an accumulation of p53. Treatment of del(5q) patients with LEN decreased p53 expression, caused by stabilization of MDM2 by inhibiting its autoubiquitination function similar to that which occurs with CRBN.\textsuperscript{175}

\textbf{RNF41.} RNF41 [ring finger protein 41, neuregulin receptor degradation protein-1 (Nrdp1), fetal liver ring finger (FLRF)] is an E3 ubiquitin ligase that has several roles.\textsuperscript{87} First, it is responsible for the ubiquitination and degradation of two additional E3 ligases, BRUCE and parkin.\textsuperscript{87,176} BRUCE is an inhibitor of apoptosis and parkin is known to be associated with disease pathogenesis of
Parkinson’s disease.\textsuperscript{87} RNF41 regulates Toll-like receptor (TLR) signaling via ubiquitination of MyD88.\textsuperscript{87} RNF41 is also involved in the steady state levels of cytokine receptors, ErbB3 and ErbB4.\textsuperscript{87,176,177} RNF41 interacts with these receptors independent of ligand binding, ubiquitinates them, and targets them for degradation and internalization.\textsuperscript{87,177} It was also found that RNF41 can regulate the steady state membrane expression of other cytokine receptors including IL3 and EpoR.\textsuperscript{87} It has since been determined that RNF41 is responsible for Jak2-associated Type I cytokine receptor ubiquitination and degradation.\textsuperscript{87} Furthermore, the regulation of cytokine receptors suggests that RNF41 may also be involved in hematopoiesis. RNF41 overexpression inhibited HSC differentiation consistent with decreased levels of EpoR and IL3.\textsuperscript{176} The role of RNF41 in Jak2-associated, Type I cytokine regulation is particularly important for this study due to its role in EpoR expression and necessity for steady state erythropoiesis.

\textbf{E3 ligases of GTPases.} Ubiquitination and proteasomal degradation of GTPases was not found until 2001.\textsuperscript{137} As RhoA, Rac1, and Cdc42 are the most well studied GTPases, the ubiquitination of these proteins was studied first. There are currently two known E3 ligases specific to RhoA and these include the HECT ligase SMURF1 (SMAD ubiquitination regulatory factor 1) and CRL3 complex (Cullin-RING ubiquitin ligase).\textsuperscript{137,166} SMURF1 activation caused a decrease in F-actin polymerization and cell motility suggesting loss of RhoA activity.\textsuperscript{166} Furthermore, loss of Cullin-3 induced actin polymerization in HeLa
cells again suggesting its activity of RhoA inhibition.\textsuperscript{166} Although Cdc42 is ubiquitinated and proteasomally degraded, the exact E3 ligases responsible for this are not yet known.\textsuperscript{137,166} The RING ligase Cullin-1 is known to ubiquitinate and cause the degradation of two known GEFs of Cdc42, FGD1 and FGD3, and therefore it is thought that proteasomal regulation of Cdc42 is accomplished primarily through regulating the GEFs and/or GAPs that interact with it.\textsuperscript{166} Interestingly, Cdc42 is thought to regulate RhoA expression by binding SMURF1.\textsuperscript{137} Rac1 is also proteasomally degraded after ubiquitination, but the ligase responsible was unknown until just recently.\textsuperscript{137} The HECT E3 ligase, HACE1, is responsible for the ubiquitination and degradation of Rac1 and was discovered after RNAi-based screening.\textsuperscript{178,179} HACE1 deletion increased Rac1 expression, however, had no effect on RhoA or Cdc42.\textsuperscript{178,179} Also of interest was the fact the HACE1 had a two-fold higher affinity for GTP bound rather than GDP bound Rac1 implicating that ubiquitination occurs after activation.\textsuperscript{178,179}

The identification of specific E3 ligases of GTPases is still in its infancy and further studies should elicit the roles of these ligases in signaling response and F-actin reorganization. We suspect IMiD induced activation of F-actin polymerization, lipid raft aggregation, and signal intermediate recruitment is accomplished through activation of the GTPases and broad E3 ligase inhibition capabilities of the IMiDs, the supporting data of which follows.
CHAPTER 2

Erythropoietin Receptor Signaling is Membrane Raft Dependent

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Introduction

Erythropoietin (Epo) is the principal regulator of red blood cell production. Upon Epo binding to its cognate receptor (R), the EpoR homodimerizes to initiate activation of the non-receptor tyrosine kinases JAK2 and Lyn, which in turn phosphorylates the receptor’s cytoplasmic tail and the signal transducer and activator of transcription 5 (STAT5). Dimerization of phospho (P)-STAT5 enables its translocation to the nucleus and binding to target gene promoters, ultimately promoting the expansion, differentiation, and survival of red blood cell precursors. The Epo signaling pathway is regulated by a balance of phosphatase and kinase activities. Lyn kinase has been shown to enhance proliferation of erythroid progenitors by increasing colony forming capacity and promoting progenitor maturation. Loss of Lyn inhibits activation of STAT5 presumably through activation of negative regulatory phosphatases, such as Src homology domain-containing phosphatase-1 (SHP-1), SHP-2, and Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP-1). Furthermore, association of Lyn with, and phosphorylation of EpoR and
STAT5, promotes activation of downstream signaling.\textsuperscript{185} Although the signaling cascade initiated by Epo and the balance of phosphatase and kinase activity has been well studied, the role of receptor localization in the plasma membrane and its effect on signal integrity has not been investigated.

The plasma membrane of hematopoietic cells contains sphingolipid and cholesterol enriched microdomains called lipid or membrane rafts.\textsuperscript{100,186} Lipid rafts represent hydrophobic, detergent-insoluble membrane fractions enriched in glycolipids and cholesterol. As a consequence, lipid rafts migrate to low density matrices upon gradient centrifugation allowing the isolation of raft membrane fractions and associated proteins.\textsuperscript{105,109} Lipid rafts are specialized membrane microdomains that cluster signaling intermediates to create focused signaling platforms that facilitate receptor-induced activation of signal transduction molecules. Rafts rapidly coalesce to form aggregates in response to cytokine stimulation or integrin engagement to optimize signal transduction.\textsuperscript{109,187-189} The clustering of rafts serves to expose proteins to a membrane environment enriched in components that amplify the signaling cascade, including kinases, scaffold and adaptor proteins, substrates, as well as redistribution of regulatory phosphatases.\textsuperscript{109,187-189} Recent investigations have shown that raft microdomains have a critical role in T-cell receptor, c-kit and integrin signaling, protein trafficking, endocytosis, as well as many other diverse cellular functions.\textsuperscript{109,121,122,190-193} In this study, we examined the role of lipid raft recruitment in EpoR signaling, receptor interaction with signaling intermediates, and EpoR signal integrity.
Results

**Epo induces raft formation and aggregation.** Lipid raft microdomains are characterized by their insoluble nature in non-ionic detergents as well as the presence of the constituent ganglioside GM-1 and double acylated proteins such as the Src-family kinase and Lyn kinase. We first investigated whether Epo affects membrane raft assembly or raft coalescence by assessing changes in membrane fraction distribution of GM-1 and Lyn kinase after Epo stimulation. Dot blot analysis of fractionated UT7 cell lysates revealed a greater than 5-fold increase of GM-1 in the detergent insoluble raft membrane fractions (fractions 1 and 2) after Epo exposure (Fig 13A), accompanied by increased raft partitioning of Lyn kinase (Fig 13B). To verify that the detergent insoluble fractions represented lipid rafts, we treated cells with a known membrane cholesterol chelating agent, methyl-β-cyclodextrin (MBCD), to disrupt raft integrity, and examined GM-1 and Lyn partitioning in membrane fractions. Treatment with MBCD abrogated partitioning of either GM-1 or Lyn into the detergent-insoluble membrane fractions, consistent with lipid raft distribution (Figs 13A and B). In T-lymphocytes, clustering of lipid rafts is an essential step in the formation of an immune synapse in response to antigen activation of the T-cell receptor. To determine if Epo promotes raft coalescence, we quantitated changes in GM-1 labeled clusters after growth factor treatment. Raft accumulation in UT7 cells after Epo stimulation increased (Fig 13C), accompanied by a significant increase.
Figure 13. Epo stimulation induces raft formation and aggregation. (A) Dot blot detection of GM-1 in UT7 cell lysates in non-raft (fractions 5, 6) and raft fractions (fraction 2) with corresponding densitometry value in controls, and after Epo or MBCD treatment. Representative blot of at least three independent experiments. (B) Western immunoblot of Lyn in raft (R) (fractions 1-2) and non-raft (NR) fractions (fractions 4-6). Treatment with Epo increased Lyn kinase incorporation into raft fractions, whereas raft disruption by cholesterol depletion with MβCD precluded Lyn incorporation. Representative western of at least three independent experiments. (C) Immunofluorescence of UT7 cells showing an increase in raft (red) accumulation after Epo exposure. (D) Immunofluorescence of UT7 cells before and after Epo stimulation showing increased raft aggregates (red) in the plasma membrane and corresponding quantitation. (E) Immunofluorescence of primary erythroid bursts showing an increase in cellular membrane raft fluorescence intensity (red). Immunofluorescence experiments were repeated at least 3 times, representative micrographs displayed.

in the mean number of raft aggregates (4.3 ± 1.4 per cell in untreated controls compared to 25.6 ± 3.2 aggregates per cell after Epo stimulation) (Fig 13D; p ≤ 0.001). The size of raft aggregates also increased after Epo treatment, with a 3.33 ± 0.11 fold increase compared to unstimulated controls (p ≤ 0.001). To
verify that the observed changes in raft dynamics in UT7 cells extends to normal erythroid progenitors, we assessed raft assembly in bone marrow erythroid bursts derived from a normal donor. BFU-E were isolated by pipetting colonies grown in methylcellulose assays after 14 days incubation. Immunofluorescence staining for GM-1 (Fig 13E) showed that mean raft fluorescence intensity in primary erythroid progenitors increased 58.4% from 72.79 ± 14/cell in unstimulated cells to 115.27 ± 14.22 after Epo treatment (p=0.01).

**EpoR co-localizes within lipid rafts.** Recruitment of the T-cell receptor into lipid rafts is a dynamic process, triggered by major histocompatibility antigen engagement. To determine if the EpoR co-localizes within raft microdomains and is influenced by ligand engagement, we assessed EpoR localization by confocal microscopy with and without Epo stimulation. EpoR rapidly co-localized with GM-1 in UT7 cells after Epo stimulation (Fig 14A, rows 1 and 2). Translocation of the EpoR to membrane rafts after Epo treatment was also confirmed in primary bone marrow erythroid bursts (Fig 14A, rows 3 and 4).

In addition to immature erythroid progenitors such as burst forming units (BFU-E), colocalization of EpoR in GM-1 raft clusters was also observed in more mature, enucleated erythroid cells after Epo stimulation (Fig 14A, bottom row). To further illustrate the recruitment of receptor to the rafts, we utilized the power of 3D rendering. Figure 14B is a representative micrograph of an unstimulated (left) and stimulated (right) UT7 cell in which the number of rafts is increased.
Figure 14. EpoR co-localizes with lipid rafts. (A) Confocal immunofluorescence of cells untreated or after Epo stimulation, lipid rafts:red, EpoR:green, DAPI/Hoechst:blue. Right panel is a merged image showing lipid raft and EpoR co-localization (yellow). UT7 cells are shown in rows 1 and 2, while human primary burst forming units are shown in rows 3 and 4, followed by a maturing, enucleated erythroid precursor in row 5. (B) Three dimensional rendering of UT7 cells either untreated (left) or after Epo treatment (right). Top two rows display isosurfacing of the rafts (red), EpoR (green), and nucleus (Dapi, blue). Dapi was removed from the middle row to further visualize association of the receptor with rafts in the second row of panels. The bottom row displays volume rendering of the same cells to illustrate membrane colocalization (yellow). (C) Quantitation of colocalization in human primary erythroid cells. Values represent mean ± SE. Immunofluorescence experiments were repeated at least 3 times, representative micrographs provided.

(red) as well as the recruitment of the receptor (green) to these domains. The bottom row in Figure 14B utilizes volume rendering to emphasize the colocalization (yellow) of the rafts and receptor on the cell surface. We used the Pearson’s coefficient to quantitate the percent of colocalization in primary BFU-E.
cells where there is a significant increase in colocalization after Epo stimulation ($p = 0.02$) (Fig 14C). EpoR membrane dynamics were further investigated by western blot analysis of membrane fractions from UT7 cell lysates isolated by gradient centrifugation. Raft (R) and non-raft (NR) fractions were pooled and separated by SDS-PAGE. EpoR was not detected in lipid rafts from unstimulated cells, but was restricted to the membrane and cytosol fractions. After 10 min of Epo exposure, the receptor translocated into raft fractions (Fig 14A), confirming that EpoR ligand engagement triggers redistribution of the receptor to membrane raft microdomains. To confirm EpoR specificity of antibody immuno-reactivity, receptor translocation was confirmed with several commercially available antibodies, including the Abcam mouse mAb (MM-0031-6G7) (Cambridge, MA), the Abcam goat polyclonal EpoR antibody, and the monoclonal A82 EpoR antibody generously provided by Amgen (Thousand Oaks, CA), each of which confirmed our findings of ligand induced raft translocation. Densitometry analysis of 2 independent experiments using all 4 of the above mentioned antibodies is presented in Figure 15A. Based on recent investigations validating the specificity of the Santa Cruz Biotechnology (Santa Cruz, CA) EpoR antibody (M-20), this antibody was used preferentially in subsequent experiments. Furthermore, although Epo signaling is known to diminish after 10 minutes, we next investigated an extended range of intervals after Epo stimulation to discern the rapidity of receptor translocation into raft fractions (Fig 15B). EpoR was recruited into the raft fractions within 1 minute of growth factor exposure,
reaching a peak at 10 minutes, followed by gradual redistribution that was completed by 60 minutes.

**Epo engagement initiates recruitment of signaling intermediates into lipid raft fractions.** Because EpoR was recruited into membrane rafts after growth factor stimulation, we investigated subcellular localization of corresponding signal effectors to determine if receptor translocation was coordinated with effector molecules to form discrete membrane platforms for receptor signaling. Immunostaining of membrane fractions for STAT5, JAK2, Lyn, and CD45 showed that Lyn and CD45 were constitutively localized in raft fractions in unstimulated cells, whereas JAK2 was absent with minimal detection of STAT5 (Fig 15C). After Epo stimulation, both JAK2 and STAT5 (principal Epo signaling proteins) translocated into raft fractions accompanied by an increase in Lyn kinase. However, CD45, a receptor tyrosine phosphatase and key negative regulator of EpoR signaling, was excluded from raft fractions and re-partitioned entirely into non-raft fractions (Fig 15C). The differential localization of CD45 after Epo stimulation suggests that growth factor activation initiates a controlled process of raft assembly and aggregation favoring the recruitment of effector molecules supporting receptor signal transduction. Furthermore, we were able to show that the activated forms of both Jak2 and Stat5, as well as the alternative Epo signaling pathway, MAPK proteins, accompanied EpoR in raft fractions after growth factor stimulation (Fig 15D).
Figure 15. Epo stimulation recruits signal effectors into raft fractions. (A) Raft fractions (R) were separated from non-raft fractions (NR) and immunoblotted for EpoR to investigate receptor translocation into rafts after Epo stimulation. Corresponding quantitation represents the mean ± SE of two independent experiments using four different EpoR antibodies. (B) Raft fractions were isolated after stimulation with Epo at the indicated time points and immunoblotted for EpoR. Results show that EpoR is recruited into rafts within 1 minute of Epo stimulation reaching maximum loading at 10 minutes, followed by gradual redistribution thereafter. Accompanying graphic quantitation of the representative experiment. (C) UT7 cells were starved overnight then treated with Epo for 10 min. After fractionation, the non-raft (NR) fractions and raft (R) fractions were pooled and immunoblotted for the indicated proteins. (D) Activated forms of Jak2, STAT5, and MAPK were also increased in the raft fractions after Epo stimulation. All westerns were repeated at least in duplicate.

**Lipid rafts are required for EpoR signaling.** Given that EpoR activation triggers formation of rafts enriched in signal effectors, we next investigated whether rafts are necessary for receptor signaling by way of raft microdomain disruption. Cholesterol depletion of UT7 cell membranes with methyl-β-
cyclodextrin (MBCD) disrupted raft integrity and completely extinguished Epo induced phosphorylation of STAT5, the primary downstream transcription factor (Fig 16A). To determine if secondary Epo signaling pathways were also affected by MBCD treatment, we probed UT7 cells for P-MAPK (mitogen-activated protein kinase). Indeed, pretreatment of cells with MBCD abrogated activation of MAPK with Epo stimulation. The PI3K/Akt pathway is not activated by Epo in UT7 cells, therefore, to investigate effects on this signaling pathway, we utilized the UT7/Epo cell line which displays Akt activation upon Epo stimulation (Fig 16B). Pretreatment with MBCD completely extinguished activation of Akt by Epo, thereby confirming that all Epo signaling pathways are impaired by raft disruption. To verify that MBCD treatment only affected signaling pathways localized to lipid rafts, we treated UT7 cells with the cell permeable phorbol 12-myristate 13-acetate (PMA), which is not directly dependent on membrane receptor activation, and induces UT7 differentiation in part through the activation of MAPK. Pretreatment of UT7 cells with MBCD prior to PMA stimulation did not affect activation of MAPK as evidenced by enzyme phosphorylation (Fig 16C). These data indicate that lipid raft integrity is essential for EpoR signaling, whereas non-receptor or non-raft signaling pathways are preserved and independent of raft integrity. To confirm that abrogation of EpoR/STAT5 signaling by MBCD is not specific to this compound, we repeated the above experiment using the cholesterol intercalating agent, nystatin, a less effective but alternative method to interfere with raft assembly and dynamics. Similar to our findings with MBCD, treatment with nystatin decreased STAT5 phosphorylation in response to Epo
Figure 16. Raft integrity is necessary for Epo-induced signaling. (A) UT7 cells were starved for 2h then pretreated with MBCD for 30min and stimulated with 3U/ml Epo for 10min; lysates were immunoblotted with the indicated antibodies. (B) UT7/Epo cells were starved for 2h then pretreated with MBCD for 30min and stimulated with 3U/ml Epo for 10min. Lysates were immunoblotted with P-Akt. The findings show abrogation of Akt phosphorylation following MBCD pretreatment. (C) UT7 cells were pretreated with MBCD for 30min, then stimulated with PMA for 30min. (D) UT7 cells were starved for 2h then pretreated with Nystatin for 30min and stimulated with Epo for 10min. Immunoblots for phospho-STAT5, STAT5, and β-actin antibodies with densitometry analysis. All westerns are representative of at least 2 independent experiments.

Raft disruption attenuates Epo-induced P-STAT5 induction in primary erythroid progenitors. To confirm raft integrity is critical to EpoR signaling in primary erythroid progenitors, we next assessed the effect of raft stimulation (Fig 16D); providing further support for the importance of lipid rafts in EpoR signal transduction.

Raft disruption attenuates Epo-induced P-STAT5 induction in primary erythroid progenitors. To confirm raft integrity is critical to EpoR signaling in primary erythroid progenitors, we next assessed the effect of raft
disruption by MBCD on Epo induced STAT5 phosphorylation by flow cytometry in bone marrow derived erythroid precursors from a normal donor. After a 2h starvation, BM-MNCs were pretreated with MBCD either with or without Epo. Cells were permeabilized and stained with antibodies to CD71, CD45, and phospho-STAT5. Epo-responsive erythroid progenitors were identified by gating on the CD45<sup>dim</sup> population of CD71<sup>+</sup> cells (Fig 17A), and phospho-STAT5 mean fluorescent intensity (MFI) was determined (Fig 17B). Treatment with MBCD significantly decreased STAT5 phosphorylation in response to Epo stimulation (Fig 5B; P=0.01). Flow histograms show a marked shift consistent with a marked reduction in phospho-STAT5 MFI (Fig 17C). These findings confirm that membrane raft integrity is critical to the fidelity of EpoR signaling in primary erythroid precursors.

**Recruitment of EpoR into lipid rafts is abrogated by Rac1 and RhoA inhibition.** Rho GTPases are key regulators of intracellular actin dynamics, and are involved in T-cell receptor trafficking into lipid rafts upon receptor stimulation.<sup>122</sup> We therefore investigated whether GTPases were also involved in EpoR recruitment into membrane rafts after Epo stimulation. UT7 cells were pretreated with a Rac1 inhibitor prior to Epo stimulation, demonstrating that inhibition of Rac1 suppressed recruitment of the receptor into raft fractions (Fig 18A). We next investigated the effects of RhoA family GTPase inhibition by pretreating cells with the Rho-associated protein kinase, ROCK, inhibitor, Y-27632; again showing that EpoR recruitment was blocked (Fig 18B). These
Figure 17. Cholesterol depletion attenuates Epo-induced STAT5 phosphorylation in primary erythroid progenitors. (A) Bone marrow mononuclear cells from a normal donor were isolated then stained with CD71:APC, CD45:FITC, and P-STAT5:PE. CD71^{Hi}/CD45^{dim} cells representing erythroid progenitors were gated. (B) Graphic comparison of geometric mean fluorescence intensities, mean ± standard error from 3 independent experiments. (C) Representative flow histogram showing shift in phospho-STAT5 fluorescence intensity in primary erythroid progenitors treated with Epo with or without MβCD.

findings suggest that Rac1 and RhoA GTPase activation is critical in the redistribution of receptor into membrane fraction upon ligand binding.

Discussion

To our knowledge, these are the first data to provide evidence that the EpoR translocates into lipid raft microdomains of the plasma membrane upon ligand engagement (Fig 14). Moreover, receptor recruitment into rafts appears
Figure 18. Recruitment of EpoR into lipid rafts is dependent on Rac1 and RhoA GTPase activation. (A) Raft fractions were isolated from UT7 cells pretreated with 100nM Rac1 inhibitor for 1hr prior to Epo stimulation then immunoblotted for EpoR with corresponding quantitation. (B) Raft fractions were isolated from UT7 cells pretreated with 100uM ROCK inhibitor (Y-27632) for 1h prior to Epo stimulation then immunoblotted for EpoR with corresponding densitometry analysis. Westerns are representative of two independent experiments.

necessary for EpoR signal fidelity and consequent activation of STAT5. In unstimulated cells, the EpoR resided largely in non-raft membrane fractions, which may serve to minimize the potential for ligand-independent interaction with signaling intermediates. Upon growth factor engagement, the receptor was recruited into lipid rafts accompanied by the incorporation of signaling effectors necessary to phosphorylate sites on the receptor tail and initiate signal transduction, including both the JAK2 and Lyn kinases, in addition to the principal downstream transcription factor, STAT5 (Fig 15). Interestingly, CD45, a transmembrane protein tyrosine phosphatase that serves to extinguish receptor signaling by dephosphorylating JAK2 and the EpoR, was constitutively localized
within membrane rafts in unstimulated cells, whereas upon stimulation with Epo, re-partitioned exclusively into non-raft fractions. These dynamic changes in CD45 partitioning should serve to optimize receptor signaling upon ligand engagement, while restricting the potential for ligand-independent effector activation in the absence of the growth factor. Moreover, these ligand induced changes in the redistribution of the EpoR and its effectors appear necessary for erythropoietin signal fidelity. Disruption of rafts by cholesterol depletion abrogated Epo-induced STAT5 phosphorylation in both UT7 cells and normal erythroid precursors (Figs 16,17), whereas non-receptor initiated activation of MAPK by PMA remained intact. Intercalation of membrane cholesterol by nystatin treatment also attenuated Epo signaling, indicating that receptor integration into rafts is critical and perhaps obligatory for EpoR signaling.

The subcellular mechanisms responsible for ligand induced changes in raft and receptor dynamics may involve G-protein controlled cytoskeletal changes. The dependence of EpoR signaling on lipid raft recruitment and assembly is analogous to the changes observed in lymphocytes after ligation of the T-cell or B-cell receptors. Within minutes of ligand engagement of the T-cell receptor, receptor subunits translocate into lipid rafts from their residence in non-raft membrane domains (Figure 15B). T-cell receptor re-distribution is controlled by G-protein coupled actin polymerization involving activation of Rac GTPases, a hematopoietic specific member of the Rho superfamily that regulates the organization, dynamics and function of the actin cytoskeleton. Conditional knock-out of Rac2 was recently shown to block early stages of
erythropoiesis in the bone marrow in murine models, suggesting that Rac2 may be a candidate molecular regulator of the observed Epo-induced changes in membrane dynamics.\textsuperscript{159,161} Our studies show that inhibition of either Rac1 or RhoA GTPases suppresses EpoR translocation into membrane raft domains. Defects in GTPase activation therefore could adversely affect receptor signaling in select pathologic conditions. In myelodysplastic syndromes, for example, Rac activation is impaired in neutrophils and CD34+ progenitors,\textsuperscript{197} accompanied by impaired lipid raft formation and a corresponding reduction in the generation of reactive oxygen species after fMLP stimulation in granulocyte-macrophage colony-stimulating factor primed neutrophils.\textsuperscript{198} Abnormalities in raft assembly in erythroid progenitors might also underlie the previously described abnormalities in EpoR signaling in MDS which warrants further investigation.\textsuperscript{49} Overall, our findings indicate that ligand engagement of the EpoR initiates dynamic changes in raft assembly and composition that bring the receptor and its effectors into spacial and temporal proximity within a discrete membrane compartment that facilitates activation of the signaling cascade. Development of strategies that enhance raft assembly and EpoR incorporation may be an attractive strategy to improve erythropoiesis in hematologic disorders with impaired erythroid response.

**Methods**

**Reagents and antibodies.** CD71:APC, P-STAT5(Y694):PE, and CD45:FITC
conjugated antibodies used for flow cytometry and anti-CD45 used for western blotting were all purchased from BD Biosciences (San Jose, CA). STAT5, Lyn, Akt, P-Jak2, and Jak2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The principal EpoR antibody used in this study was purchased from Santa Cruz Biotechnology (M-20). To confirm immunospecificity of EpoR localization (Fig 15A) we also included Abcam mouse mAb (MM-0031-6G7), Abcam goat polyclonal, and Amgen (Thousand Oaks, CA) A82 EpoR antibodies. ROCK inhibitor, Y-27632 dihydrochloride monohydrate, cholera toxin B (CTB) HRP conjugate, methyl-beta-cyclodextran, Nystatin, and PMA were purchased from Sigma-Aldrich (St. Louis, MO). P-MAPK, MAPK, and anti-P-STAT5 (Y694) for westerns were purchased from Cell Signaling Technology (Danvers, MA). P-Akt, Alexa Fluor® 488 goat anti-rabbit IgG, and Vybrant® Lipid Raft Labeling Kit were ordered from Invitrogen (Carlsbad, CA). Recombinant human Epo (Epo) was purchased from Stemcell Technologies (Vancouver, BC, Canada). Rac1 Inhibitor was purchased from EMD Millipore (Billerica, MA).

**Cell lines and bone marrow cultures.** The human leukemic cell line, UT7, was obtained from ATCC (Gaithersburg, MD). UT7 cells were maintained in α-MEM medium supplemented with 20% fetal bovine serum (FBS), 1% penicillin/streptomycin solution, and 5ng/ml GM-CSF. UT7/Epo cells were maintained in IMDM medium supplemented with 10% FBS, 1% penicillin/streptomycin solution, and 1U/mL Epo. After overnight starvation, cells
were stimulated with Epo at a concentration of 3U/mL. For Rac1 and ROCK inhibitor experiments, cells were pretreated for 1h with 100nM and 100µM, respectively, before stimulation with Epo. Low-density mononuclear cell (MNC) fractions were isolated from heparinized bone marrow aspirates from healthy volunteers purchased from Lonza Walkersville Inc. (Walkersville, MD) using standard density gradient centrifugation over Ficoll-Paque Plus (GE Healthcare, Little Chalfont, United Kingdom), followed by washing and resuspension in Iscove’s Modified Dulbecco Medium (IMDM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

Erythroid progenitors at the burst-forming unit–erythroid (BFU-E) stage of differentiation were grown in cytokine-defined IMDM, similar to previous studies. Briefly, 2 X 10^5 MNC per mL were plated in Complete Methocult® medium (StemCell Technologies, Vancouver, BC, Canada) supplemented with 10% FBS and 3 U/mL erythropoietin. Plates were incubated at 37°C in a 5% CO₂ air mixture in a humidified incubator for 14 days. BFU-E were identified using an inverted microscope, aspirated by pipette, washed twice in PBS then resuspended in IMDM for immunofluorescence studies.

**Immunoblotting.** Cells were starved in 0.5% FBS containing medium for 2h prior to 30 min pre-incubation with 10mM MBCD or 50µg/ml nystatin, or stimulation with 3U/ml Epo (10 min) or 100ng/ml PMA (30 min). For RAC and ROCK inhibitor experiments, cells were pretreated for 1h prior to Epo stimulation. Cells were washed 3x in cold PBS and lysed in 1X RIPA buffer containing 250µM
NaVO₄, 2µg/ml aprotinin, 2µg/ml leupeptin, 0.2µg/ml pepstatin A, and 500µM PMSF. Sample buffer was added to cell lysates and 100µg of protein was separated using SDS-PAGE. Proteins were transferred to PVDF membranes and immunoblotted with the indicated antibodies. Membranes were developed using ECL or ECL Plus according to manufacturer’s protocols (GE Healthcare, Piscataway, NJ).

Flow cytometry. Bone marrow from normal donors was purchased from Lonza (Walkersville, MD). BM-MNCs were isolated using Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) and starved for 2h in 0.5% FBS containing IMDM medium. The cells were then pretreated with 10mM MBCD for 30min and stimulated with 3U/ml Epo for 10min. They were immediately washed 3x in cold Staining Buffer (BD Biosciences San Jose, CA), fixed for 10min at 37°C in Cytofix (BD), then permeabilized for 30min on ice with Perm Buffer III (BD). Cells were stained with CD71:APC, CD45:FITC, and P-STAT5:PE conjugated antibodies. Cells were washed with Staining Buffer and analyzed on a FACScalibur flow cytometer. Primitive erythroid cells were captured in CD71^{Hi} and CD45^{Dim} gated population.

Lipid raft isolation. Lipid Rafts were isolated as previously described.¹⁰⁵,¹⁰⁸ Briefly, UT7 cells were washed 2x with cold PBS then lysed in 0.75% Triton X-100 in TNE Buffer [TNE buffer composed of 25mM Tris pH7, 150mM EDTA, 1mM DTT, 150mM NaCl, and 1 Complete EDTA-free protease
inhibitor tablet from Roche (Indianapolis, IN) per 20ml buffer]. Cells were passed through a 27G needle several times and incubated on ice for 5min. Two hundred microliters of lysate were mixed with 400µL of 60% Optiprep (Sigma-Aldrich, St. Louis, MO) and pipetted into an ultracentrifuge tube. Decreasing percentages of Optiprep (35%, 30%, 25%, 20%, and 0%) were loaded on top of each other and the tubes were spun at 20000rpm for 20h in a Beckman Coulter (Fullerton, CA) Optima L-90K ultracentrifuge. Fractions were pipetted off one by one and used for dot and western blotting.

**Dot blots.** Five or ten microliters of fractionated cell lysates were pipetted directly onto nitrocellulose membrane. The membranes were allowed to dry then washed briefly in PBS. They were then blocked in 0.3% Tween20 PBS for 30min and incubated in cholera toxin B:HRP conjugated antibody overnight. The blots were washed 3x in 0.3% Tween20 PBS and developed with ECL.

**Immunofluorescence.** Starved UT7 cells (0.5% FBS supplemented α-MEM medium) were stained with Vybrant® Lipid Raft Labeling Kit according to manufacturer’s protocol, treated with 3U/ml EPO for 10min at 37°C and fixed with Cytofix (BD Biosciences San Jose, CA) for 10m at 37°C. Cells were then cytopspun and stained with EpoR antibody at a 1:50 dilution for 1hr at 37°C, washed in PBS and stained 1:500 with Alexa Fluor® 488 goat anti-rabbit IgG for 1hr at 37°C. Cells were then mounted using ProLong® AntiFade reagent with DAPI (Invitrogen, Carlsbad, CA) and cover slip placed on top. Micrographs were
taken using a Leica TCS SP5 AOBS Laser Scanning Confocal microscope (Leica Microsystems, Germany). BFU-E colonies isolated from progenitor cultures from a normal donor were washed 2X then starved in 0.5% FBS supplemented IMDM medium for 2h. They were then stained with EpoR and Alexa Fluor® 488 goat anti-rabbit IgG as above. The cells were then washed and stained with Vybrant® Lipid Raft Labeling Kit according to manufacturer’s protocol. Cells were resuspended in 1ml medium and stained with 1µg/ml Hoechst stain (Invitrogen, Carlsbad, CA). Micrographs of the untreated cells were taken by confocal microscopy then 3U/ml of Epo was added to the plate and micrographs from stimulated cells were taken 5-20min after Epo treatment.

**Immunofluorescence image analysis.** Photomicrographs were obtained using a Leica TCS SP5 AOBS laser scanning confocal microscope at zoom through a 20X/0.5NA or 63X/1.40NA Plan Apochromat oil immersion objective lens (Leica Microsystems, Germany). 405 Diode, Argon 488, and HeNe 543 or 594 laser lines were applied to excite the fluorophores and tunable emissions were used to minimize crosstalk between fluorochromes. Gain, offset, and pinhole settings were identical for all samples within the treatment group. Image sections were collected at either 0.2 µm (for 3D reconstructions) or at 0.5µm were captured with photomultiplier detectors and maximum projections were prepared with the LAS AF software version 2.1.0 (Leica Microsystems, Germany). In some cases, 4X zoom was applied when acquiring images. Intensity and aggregate analysis were performed using Image Pro Plus version 6.2 (Media
Identical threshold settings and measurement parameters were used to generate the mean intensity and area data. Aggregates were defined as an object within the cell that has an intensity value of at least 20 and an area between 3 and 600 pixels. Three dimensional isosurface renderings were prepared with Imaris software version 5.5.3 (Bitplane Inc., Zurich, Switzerland).

**Statistical analysis.** Numerical data are expressed as mean ± standard error of the mean. Statistical analyses were performed using the Student t test (2-tailed for equal variances). P values < 0.05 were considered significant. Pearson's correlation analysis for colocalization was performed using Definiens Developer version 1.5 (Definiens AG, Munich, Germany).
CHAPTER 3
Lenalidomide induces Lipid Raft Formation and F-Actin Polymerization

Introduction

Bone marrow progenitors from patients with MDS display diminished activation of STAT5 in response to Epo stimulation despite normal or elevated levels of endogenous serum Epo and similar EpoR membrane density compared to normal counterparts. Our laboratory reported that the EpoR resides within plasma membrane microdomains known as lipid, or membrane rafts, which is critical to EpoR signal competence (Chapter 2). Epo induced the formation and aggregation of lipid rafts, as well as the recruitment of key signaling intermediates such as EpoR, Jak2, STAT5, and Lyn kinase. Furthermore, receptor engagement of the Epo ligand triggered the translocation of the signal attenuating transmembrane tyrosine phosphatase, CD45, to non-raft domains, ultimately potentiating signal capacity. Disruption of rafts by membrane cholesterol depletion inhibited Epo induced activation of STAT5 in both erythroid cell lines and primary bone marrow erythroid progenitors, thereby confirming the critical role of raft integrity in cellular Epo response. Furthermore, inhibition of Rho and Rac GTPases, important regulators of the actin cytoskeleton, blocked recruitment of EpoR into the raft fractions, indicating a critical role for these proteins in the coordination of EpoR membrane domain localization.
GTPases are activated by immunomodulatory agents (IMiDs) which in turn trigger assembly of the immune synapse in T- and NK-cells. LEN enhances erythroid progenitor expansion, potentiates in vitro colony forming capacity in response to Epo, and augments Epo induced receptor signaling through mechanisms that have not as yet been characterized. Approximately 75% of lower risk, del(5q) MDS patients will respond to LEN acquiring red blood cell transfusion independence, hemoglobin normalization, and decrease in the del(5q) clone. This occurs through direct suppression of the del(5q) clone via inhibition of the haplosufficient cell cycle regulatory phosphatases, Cdc25c and PP2A, resulting in G2/M cell arrest and apoptosis. In non-del(5q) MDS, erythropoietic rescue occurs in approximately 25% of patients in the absence of cytotoxicity to the MDS clone, as evidenced by a decrease in bone marrow apoptotic fraction in responding patients, suggesting erythropoietic potentiating effect. In their report, Ebert et al. showed that LEN treatment restored expression of the underexpressed erythroid differentiation gene set in responding patients, indicating that LEN can improve inherent limitations in EpoR transcriptional response in MDS. We hypothesize that there is a decrease in lipid raft density in MDS patients and that LEN acts at a proximal level in EpoR signaling by recruiting signaling intermediates and EpoR into aggregated, active signaling raft platforms. And, that these effects are dependent on the activation of the Rho kinase, ROCK.
Results

**LEN induces lipid raft formation.** We previously showed that treatment of the erythroleukemia cell line, UT7, with recombinant human erythropoietin (rhEpo) stimulated rafts within minutes of growth factor exposure.\(^{200}\) We next investigated the effects of LEN on raft formation and aggregation. To assess this, we treated UT7 cells with 1µM LEN for 1h. Lipid rafts were isolated by ultracentrifugation and fractions were dot blotted for GM-1 detection (Figure 19A). GM-1 is a raft constituent ganglioside and its fractionation and membrane localization is used as a marker of lipid rafts.\(^{105,131,132}\) The observed increase in GM-1 positive membrane fractions (fraction 2) indicates that LEN treatment increased membrane lipid rafts, and this increase was more than that observed with rhEpo stimulation (Figure 19A). To confirm this finding, we next analyzed raft aggregation by confocal microscopy in UT7 cells after LEN treatment. We found a marked increase in the number and size of lipid rafts as ascertained by GM-1 detection (Figure 19B). These results demonstrate that LEN is able to stimulate raft formation and raft aggregation in the absence of cytokine or receptor stimulation.

**LEN recruits signal effectors into raft fractions.** Our prior studies showed that in addition to inducing raft formation and aggregation, rhEpo induced the recruitment of EpoR and signal intermediates Jak2, STAT5, and Lyn
Figure 19. LEN induces the formation of membrane lipid rafts. (A) Dot blot detection of GM-1 membrane fractionation in UT7 cells treated with 1U/ml erythropoietin or 1µM LEN for 1hr. Rafts are located in fraction 2 and non-rafts fractions are in 4-6. (B) Immunofluorescence of UT7 cell rafts in red showing a marked accumulation after LEN treatment.

kinase into the raft platforms. Treatment with rhEpo also sequestered the negative regulator and transmembrane protein, tyrosine phosphatase CD45, out of the raft fractions, thereby potentiating fidelity of the EpoR signal. To determine whether LEN treatment effected raft constituents, we treated UT7 cells and isolated both the raft fractions (fraction 1-3) and non-raft fractions (fractions 4-6) after ultracentrifugation (fractions were also confirmed by dot blot, data not shown). These fractions were then probed by western blot (Figure 20). We found that LEN readily induced the recruitment of EpoR into lipid raft fractions after 1hr of drug exposure (Figure 20). We utilized the Santa Cruz (M-20) EpoR
Figure 20. LEN treatment induces recruitment of EpoR and signaling effectors into lipid rafts. Western blot of fractionated rafts (red boxes) and non-raft fractions. LEN induces recruitment of EpoR, Jak2, and Stat5 into the raft fractions while displacing the negative phosphatase regulator, CD45. Lyn kinase serves as a marker for lipid raft fractionation, although no redistribution was observed after LEN treatment.

antibody in these experiments based on previous studies, including ours, validating its specificity. Furthermore, both Jak2 and STAT5 showed increased fractionation with GM-1 after LEN treatment indicating recruitment of signaling effectors into discrete signaling platforms, similar to that described after T-cell activation. Additionally, we found that LEN treatment also partially redistributed CD45 out of the raft fractions further promoting signal efficiency. Although Lyn kinase fractionation did not change, its partitioning is another method by which lipid raft fractions (fractions 1-3) may be ascertained.
**ROCK inhibition blocks LEN Induced raft formation.** We previously demonstrated that rhEpo induction of lipid rafts was dependent on the Rac GTPases. Inhibition of both the Rho kinase, ROCK, and Rac GTPase inhibited recruitment of EpoR into the raft fractions after Epo stimulation. We wished to determine whether ROCK was similarly involved in LEN induced raft formation. UT7 cells were treated with LEN either with or without pretreatment with 100µM ROCK inhibitor, Y-27632, for 30m. Pretreatment of cells with Y-27632 inhibited the induction of lipid rafts by LEN as shown by GM-1 dot blot detection (Figure 21A). These data were further confirmed by confocal microscopy (Figure 21B), and suggest that the induction of rafts by LEN is dependent on the ROCK kinase.

![Figure 21](image)

**Figure 21.** LEN induced raft formation is ROCK dependent. (A) Dot blot detection of GM-1 in UT7 cells treated with LEN either with or without ROCK inhibitor, Y-27632 (ROCKi), pretreatment. Rafts are located in fractions 1 and 2, while non-raft fractions are 4-6. (B) Immunoflorescence of rafts (red), nuclei (blue), and merged image showing inhibition of LEN induced raft formation with ROCKi pretreatment.
**ROCK inhibition blocks LEN induced F-actin polymerization.** The immune synapse in T and NK cells is formed through extensive lipid raft aggregation initiated by F-actin polymerization, a process that is regulated by the Rho GTPase, ROCK, and LIM kinase (LIMK).\textsuperscript{127-129} Rho activates actin polymerization through the Wiscott-Aldrich Syndrome protein (WASp), which in turn activates the Arp2/3 complex proteins that are responsible for promoting actin polymerization.\textsuperscript{157} Furthermore, Rho activates the myosin light chain (MLC) promoting actin assembly, while ROCK inactivates the MLC negative regulatory phosphatase.\textsuperscript{127,143,147,151} Lastly, LIMK kinase phosphorylates cofilin. When cofilin is phosphorylated, it is no longer able to bind to actin and prevent polymerization.\textsuperscript{150} Disruption of the Rho/ROCK/LIMK pathways resulted in decreased raft accumulation and recruitment of the T-cell receptor, with consequent impaired immunological synapses.\textsuperscript{127-129} We sought to determine whether LEN induced actin polymerization in UT7 cells to foster raft assembly and whether this process was similarly dependent on ROCK. Cells were treated and stained with phalloidin and analyzed by confocal microscopy (Figure 22). LEN treatment induced actin filament polymerization that was inhibited by pretreatment with the ROCK inhibitor, Y-27632. Analogous to the formation of the immunological synapse, ROCK dependent F-actin assembly is likely responsible for the coalescence of rafts and EpoR signaling components in erythroid cells.
Lenalidomide induces raft formation in MDS erythroid progenitors.

Previous reports have shown that fLMP stimulated granulocyte–macrophage colony stimulating factor (GM-CSF) primed MDS neutrophils have decreased raft density associated with decreased production of reactive oxygen species.\textsuperscript{198}

![Figure 22. LEN induces actin polymerization that is blocked by ROCK inhibition. Phalloidin (green) was used to detect actin polymerization. LEN treatment induced actin polymerization, but was inhibited by pre-treatment with ROCK inhibitor (ROCKi). DAPI (blue), last panel is merged image.](image)

Furthermore, Rac activation is reported to be impaired in both neutrophils and CD34+ progenitor cells in MDS.\textsuperscript{197} Therefore, we sought to determine whether an impairment in lipid raft assembly limits membrane raft density in primary MDS erythroid progenitors to contribute to diminished Epo responsiveness.\textsuperscript{198} Primary bone marrow mononuclear cells were isolated from 11 non-del(5q) MDS patients consented on IRB approved research protocols and from 3 normal donors
purchased from Lonza Walkersville. Cell were treated with LEN then cytospun and stained with CD71 and c-Kit antibodies, as well as for lipid rafts. Erythroid progenitor cells were identified as dual CD71 and c-Kit+; the number of raft clusters was determined by confocal microscopy. Mean number of membrane raft clusters in MDS erythroid progenitors was decreased compared to normal volunteers (p=0.129) (Figure 23). This deficiency was partially rescued with LEN treatment. Although we did not reach statistical significance, we suspect that if we increased the number of samples used, that statistical significance may be achieved. These results provide a novel mechanism of action for LEN in primary non-del(5q) MDS cells and warrants study in a larger data set.

Figure 23. LEN induces rafts in deficient non-del(5q) MDS primary erythroid progenitors. Immunofluorescence of raft density in primary normal and non-del5q MDS bone marrow erythroid progenitors (CD71+, c-Kit+). DAPI (blue), CD71 (green), c-kit (pink), rafts (red). Primary MDS progenitors show decreased raft clusters compared to normal controls, however, raft formation is induced with LEN treatment.
Discussion

The data shown here provide new insight as to abnormalities in the EpoR signaling platform that may underlie the impaired responsiveness of erythroid precursors to Epo in MDS. We show that EpoR signal fidelity is dependent upon proper and adequate lipid raft assembly in the plasma membrane. In MDS erythroid precursors, we found that raft formation is deficient, and importantly, that LEN augments Epo-induced erythroid expansion. Our findings reveal that LEN is able to promote lipid raft formation and the recruitment of EpoR into the raft microdomains. Additionally, Jak2 and STAT5 are recruited to the rafts compartments while the tyrosine phosphatase CD45 is re-partitioned out of raft fractions upon LEN exposure. LEN induced raft assembly that was dependent upon F-actin polymerization, a process which was dependent on the Rho kinase, ROCK. GTPases are known to coordinate reorganization of the actin cytoskeleton, which is responsible for raft coalescence and the formation of the immune synapse in T-cell activation.\textsuperscript{127-129}

The actin cytoskeleton has important roles in cell proliferation and differentiation, and aberrancies in actin polymerization have been implicated in the disease pathogenesis of hematological malignancies. Recent findings have shown that the unconventional Rac activating guanine nucleotide exchange factor (GEF), DOCK4, is decreased in MDS patients compared to age-matched controls. DOCK4 is a member of the CDM (C. elegans Ced-5, mammalian DOCK180 and D. melanogaster myoblast city) family of proteins which are known regulators of adheren junctions and cell migration. The \textit{DOCK4} promoter
is hypermethylated in MDS causing gene silencing and decreased protein expression.\textsuperscript{199} Of note, \textit{DOCK4} is localized to chromosome 7q31, and deletions or translocations involving this site are associated with poor prognosis in MDS and AML. Recent data presented at the 2012 American Society of Hematology annual meeting showed that silencing of \textit{DOCK4} in MDS was associated with diminished F-actin polymerization.\textsuperscript{203} Furthermore, decreased DOCK4 was associated with increased erythrocyte fragility, whereas knockdown of \textit{DOCK4} in primary progenitor cells led to lineage specific apoptosis of erythroid progenitors, features shared by MDS progenitors. These findings provide a plausible pathobiological rationale for the ineffective erythropoiesis in MDS in which intrinsic cytoskeletal abnormalities arising from decreased DOCK4 initiated polymerization of actin impairs lipid raft assembly and growth factor receptor incorporation. As a consequence, cytokine signal capacity and cell survival are diminished. Rac GTPase dependent raft integrity, which in our investigations is partially rescued by LEN, further supports this notion. The effects of LEN on DOCK4 expression and activity warrants further investigation.

Although LEN is known to activate GTPases, the mechanism by which this occurs is unknown.\textsuperscript{128} Recent findings demonstrate that IMiDs bind to the cereblon E3 ubiquitin ligase complex to inhibit ligase function, which appears responsible for the teratogenicity of thalidomide as well as the anti-proliferative effects of both LEN and thalidomide in multiple myeloma. Furthermore, we recently reported that LEN inhibits the ligase activity of MDM2.\textsuperscript{204} Inhibition of MDM2 auto-ubiquitination stabilizes the protein, permitting binding to and
degradation of p53 in del(5q) clones.204 These findings suggest that LEN may have broader E3 ligase inhibitory effects. It is possible that LEN may activate GTPases via inhibition of the E3 ligases responsible for their degradation. Currently, several ligases are recognized to ubiquitinate RhoA, including SMAD ubiquitination regulatory factor 1, SMURF1 and the CRL3 complex (Cullin-RING ubiquitin ligase). The HECT ligase, HACE1, has recently been reported to be involved in the ubiquitination and degradation of Rac1. Additional investigations are warranted to determine whether these ligases are also inhibited by LEN. These experiments will determine whether E3 ligase inhibition is responsible for GTPases activation and consequent actin cytoskeletal reorganization that augments EpoR signaling by modulating raft assembly and composition.

Methods

Reagents and cells. UT7 cells were maintained in alpha-MEM supplemented with 20% FBS, 1% penicillin/streptomycin solution, and 5ng/ml GM-CSF in a humidified incubator with 5% CO2. Bone marrow mononuclear primary cells were isolated from 11 MDS patients consented on IRB approved protocols using Ficoll-Paque (GE Healthcare, Little Chalfont, UK) method and from 3 normal donors purchased from Lonza Walkersville Inc (Walkersville, MD). LEN was purchased from Fisher Scientific (Pittsburgh, PA). CT-B:HRP was purchased from Sigma-Aldrich (St. Louis, MO) EpoR, Jak2, Stat5, CD71, c-Kit, and Lyn antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz,
CD45 antibody was purchased from BD Biosciences (San Jose, CA). Secondary antibodies were purchased from Life Technologies Corporation (Invitrogen, Carlsbad, CA). ProLong® Anti-fade reagent with DAPI was purchased from Life Technologies (Invitrogen). ROCK inhibitor, Y-27632 dihydrochloride monohydrate, was purchased from Sigma-Aldrich.

**Lipid raft isolation.** Lipid rafts were isolated as previously described.\(^{200}\) Briefly, cells were lysed in 0.75% Triton X-100 in TNE buffer [25mM Tris pH7, 150mM EDTA, 1mM DTT, 150mM NaCl, and 1 Complete EDTA-free protease inhibitor tablet from Roche (Indianapolis, IN) per 20ml buffer]. Cells were then passed through a 27G needle and left on ice for 5min. Lysates were then pipetted below a decreasing concentration gradient of Optiprep purchased from Sigma-Aldrich. Samples were ultracentrifuged at 20000rpm for 20h in a Beckman Coulter (Fullerton, CA) Optima L-90K ultracentrifuge. Fractions were pipetted off one by one and used for either western blotting and/or dot blotting.

**Western blotting.** Fractions isolated after raft isolation were resolved by SDS-PAGE then transferred to PVDF membranes. The membranes were blocked for 30 min in 5% dry milk solution in PBST (PBS with 0.1% Tween 20) and incubated with the indicated antibodies. Membranes were developed using ECL or ECL+ according to manufacturer’s protocol (GE Healthcare, Little Chalfont, UK).
**Dot blotting.** Five microliters of each fraction isolated from ultracentrifugation was pipetted onto a nitrocellulose membrane. The membrane was then washed in PBS and blocked for 30 min in 0.3% PBS-Tween20 solution. Membranes were then incubated with CT-B:HRP overnight then washed three times in PBS with 0.3% PBS Tween20. Membranes were developed using ECL according to manufacturer’s protocol.

**Immunofluorescence.** Raft immunofluorescence was performed as previously described. Briefly, treated cells were stained with Vibrant Lipid Raft Labeling kit (Invitrogen) per manufacturer’s protocol, then cyto spun at 450 rpm for 5 min. Slides were then fixed with BD Cytofix (BD Biosciences) for 10 min at 37°C. Slides were washed in PBS and a drop of ProLong® Anti-fade reagent with DAPI was added with a cover slip. Micrographs were taken using a Leica TCS SP5 AOBS Laser Scanning Confocal microscope (Leica Microsystems, Germany). For F-actin staining, cells were treated, then fixed and cyto spun. Slides were permeabilized in 0.5% Triton-X for 5 min at room temperature then washed and blocked in 2% BSA-PBS. Cells were then stained with Alexa Fluor 488 phalloidin according to manufacturer’s protocol (Invitrogen). Cells were washed, then ProLong® Anti-fade reagent with DAPI was added with a coverslip, and micrographs were taken on the Leica TCS SP5 AOBS Laser Scanning Confocal microscope. For the primary cell immunofluorescence experiments, rafts were stained as described above. Before adding DAPI, cells were blocked and stained with CD71 and c-Kit antibodies for 1 h at room temperature. Slides
were then washed and incubated in secondary antibody (1:1000) for 1 hr at room temperature. Micrographs were taken by confocal microscopy.
CHAPTER 4

Lenalidomide Stabilizes EpoR Expression through Inhibition of the E3 Ubiquitin Ligase, RING Finger Protein 41 (RNF41)

A note to the reader: Portions of this work have been previously published in the journal Blood, Basiorka et al. 2011. 118:2382a and Basiorka et al. 2012 120(21):3455a, and USF Honors College Undergraduate Thesis, Ashley Basiorka, 2012, and have been reproduced here with permissions.

Introduction

Lenalidomide (LEN), restores defective erythropoiesis and red blood cell transfusion independence in approximately 25% of non-del(5q) MDS patients. Gene expression profiling performed by Ebert et. al. showed that LEN responders displayed inherently lower expression levels of erythroid specific genes that were restored by treatment with LEN. Our investigations showed that LEN acts to enhance EpoR signal capacity to increase transcriptional response to Epo ligand receptor engagement. The latter is achieved at least in part through LEN’s potentiating effect on lipid raft assembly accompanied by recruitment of the EpoR and key signaling intermediates into the raft microdomains (Chapter 3).

Recent investigations revealed that both thalidomide and LEN bind to and inhibit the function of the cereblon E3 ubiquitin ligase complex, which has been implicated in LEN antiproliferative effects in multiple myeloma, and the teratogenicity of thalidomide. Our laboratory and colleagues recently reported that LEN binds to and inhibits the function of another E3 ubiquitin ligase, the murine double
minute-2 protein (MDM2). LEN inhibits auto-ubiquitination of MDM2 to stabilize the protein and foster its binding to and degradation of p53. Because EpoR turnover is regulated by ubiquitination and proteasomal degradation, we evaluated the effects of LEN on the E3-ubiquitin ligase, RNF41, which regulates steady state or ligand independent, Janus kinase (JAK2) associated Type I receptor internalization. We hypothesized that LEN upregulates JAK2/EpoR expression through inhibition of RNF41, thereby enhancing JAK2 competent receptor signaling.

Results

Lenalidomide upregulates EpoR protein expression. To determine the effect of LEN on EpoR expression, UT7 cells were treated with increasing concentrations of LEN for 1hr. Western immunoblot showed that LEN increased EpoR protein expression in a dose dependent manner (Figure 24A). We next treated cells over an extended period and found that upregulation of EpoR after LEN exposure doubled within 1hr of treatment (Figure 24B), and continued to increase through 8hr of incubation, showing no significant decay as late as after 24 hours of drug exposure (Figure 24C, densitometry analysis Figure 24D). To determine whether EpoR upregulation was transcriptionally mediated, EpoR gene expression was assessed by real time Q-PCR. We found no change in EpoR mRNA expression after LEN treatment, indicating that receptor protein upregulation is a post transcriptional event (Figure 24E). To investigate whether LEN had similar
Figure 24. LEN increases EpoR expression. (A) Western blot of UT7 cells treated with increasing concentrations of LEN for 1hr showing a dose-dependent increase in EpoR expression by the immunomodulatory agent, lenalidomide (LEN). (B) Densitometry analysis. (C) Western blot of UT7 cells treated with 1µM LEN over the indicated time intervals showing an increase in EpoR protein expression as early as 1hr with continued increase up until 8hr after treatment. (D) Densitometry analysis. (E) Relative expression of UT7 EpoR mRNA detected by Q-PCR showing no change in transcription indicating that LEN increases EpoR expression at the protein level.

receptor modulating effects in primary erythroid progenitors, bone marrow mononuclear cells (BM-MNC) were isolated from three normal donors and changes in EpoR expression was assessed by quantitative fluorescence microscopy in erythroid precursors identified by CD71 expression (Figure 25A). We confirmed that LEN induced a statistically significant increase in EpoR expression in normal, primary erythroid progenitors after 1h of drug exposure (Figure 25B). Mean
Figure 25. LEN induces EpoR expression in primary erythroid progenitors. (A) Representative immunofluorescent micrographs of three primary normal BM-MNC erythroid progenitors. Erythroid progenitors were identified as CD71+. Dapi (blue), CD71 (green), EpoR (red), and merged image. (B) Mean fluorescence intensities (MFI) +/- standard error showing an increase in EpoR expression in erythroid progenitors after LEN treatment (p=0.003). N=total number of cells analyzed from all donors.

fluorescent intensity (MFI) of untreated erythroid progenitors was 1043.5 +/- 32.5 (SE) vs 1216.6 +/- 51.7 for cells treated with 1µM LEN for 1 hr (p=0.003).

**LEN stabilizes EpoR expression.** Our findings that LEN treatment yielded a sustained cellular increase in EpoR receptor expression suggested that LEN may act through suppression of receptor turnover. To determine whether LEN increased the stability of EpoR protein, we first treated cells with cyclohexamide (CHX) to
inhibit new protein synthesis. UT7 cells were treated with 1µM CHX for 24hr either with or without LEN (co-treated for 1hr after 24hr CHX pretreatment), and lysates collected at the indicated time points over a 72hr period. Western blot was performed to investigate the levels of EpoR at each time point. Treatment of UT7 cells with CHX showed approximately a 50% reduction in EpoR expression at 56hr, however, addition of LEN markedly extended the half-life of EpoR to beyond 72hr (Figure 26). These data demonstrate that LEN stabilizes the EpoR protein, to increase cellular density of signaling competent receptors.

![Western blot of UT7 cells treated with cyclohexamide (CHX) either with or without LEN treatment. Treatment with LEN increased EpoR stability changing the half-life from approximately 56hr to out past 72hr.](image)

Figure 26. LEN increases EpoR stability. Western blot of UT7 cells treated with cyclohexamide (CHX) either with or without LEN treatment. Treatment with LEN increased EpoR stability changing the half-life from approximately 56hr to out past 72hr.

**Cytokine receptor induction by LEN is limited to Type I cytokine receptors.** To determine if the effects of LEN on receptor turnover are restricted to Type 1 cytokine receptors, we examined the effects of LEN on cellular expression of IL3-R (Type 1) and c-Kit (Type 2). LEN upregulated IL3-R expression in a concentration-dependent manner, whereas c-Kit expression was unchanged, confirming Type 1 receptor specificity (Figure 27).
LEN inhibits the E3 ubiquitin ligase activity of RNF41. Recent investigations have shown that steady state EpoR turnover is regulated through the E3 ubiquitin ligase, RNF41. RNF41 regulates ligand independent expression levels of Jak2 associated, Type I cytokine receptors via substrate ubiquitination and targeted proteasome degradation. We first confirmed that RNF41 bound to EpoR/Jak2 complexes after LEN treatment by protein immunoprecipitation (IP) followed by EpoR and Jak2 immunoblot (IB). EpoR:RNF41 binding increased in a concentration dependent fashion with LEN treatment (Figure 28A). Additionally, IP of EpoR followed by IB of RNF41 showed similar results (data not shown). To investigate the effects of LEN on RNF41 function, we assessed protein specific ubiquitination after proteasomal inhibition with bortezomib followed by LEN treatment. IP of RNF41 followed by ubiquitin IB showed that LEN inhibited RNF41 auto-
ubiquitination in a concentration-dependent fashion, therefore mirroring the effects on receptor:RNF41 association (Figure 28B). Drug inhibition of the E3-ubiquitin ligase auto-ubiquitination resulted in cellular accumulation of RNF41 expression with corresponding increased association with EpoR and Jak2 (Figure 28A and C), paralleling the decrease in EpoR ubiquitination, suggesting that the E3-ubiquitin ligase inhibitory effects of LEN extends to RNF41 (Figure 28D).

Figure 28. LEN inhibits RNF41 ubiquitin ligase function. (A) Immunoprecipitation (IP) of RNF41 in UT7 cells treated with LEN at indicated concentrations for 1hr. There is a dose dependent increase in co-immunoprecipitation of EpoR and Jak2 with RNF41 after LEN treatment. (B) RNF41 was immunoprecipitated then immunoblotted (IB) for ubiquitin. Bortezomib was used to block proteasomal degradation. LEN decreases the ubiquitination of RNF41 in a dose dependent manner. (C) RNF41 protein expression levels increase in total cell lysates of UT7 cells treated with LEN for 1hr at the indicated concentrations corresponding with decreased RNF41 ubiquitination. (D) Ubiquitination of EpoR decreases with LEN treatment [(IP) EpoR, (IB) ubiquitin] via inhibition of RNF41 ligase activity.
**RNF41 overexpression abrogates LEN-induced upregulation of EpoR.**

To confirm that RNF41 is the principal target of LEN responsible for EpoR stabilization, we transfected HEK293T cells with EpoR and/or RNF41 expression vectors using the calcium phosphate method. Steady state EpoR expression was lower in EpoR/RNF41 cells compared to cells transfected with EpoR alone (Figure 29). Moreover, EpoR upregulation by LEN was abrogated in EpoR/RNF41 cells indicating that cellular RNF41 is a critical determinant of EpoR upregulation by LEN.

![Western blot and corresponding densitometry analysis](image)

Figure 29. Overexpression of RNF41 blocks LEN-induced increase in EpoR expression. Western blot and corresponding densitometry analysis of UT7 cells transfected with EpoR (pMET7-EpoR) or EpoR and RNF41 (pMet7-RNF41) showing a decrease in steady state EpoR, as well as inhibition of LEN-induced EpoR, with RNF41 overexpression.
RNF41 expression is decreased in LEN responsive MDS primary erythroid cells. To determine the effects of LEN on RNF41 expression in vivo, we performed immunohistochemistry on 18 (6 LEN responders, and 12 non-responders) bone marrow biopsies from non-del(5q) MDS patients and stained for RNF41 and the erythroid marker spectrin (Figure 30A). By assessing cellular expression profiles in bone marrow biopsies obtained before and after LEN treatment, we were able to assess the relationship between cellular RNF41 level in erythroid precursors and clinical erythroid response. Relative expression of RNF41 in erythroid precursors at baseline was lower in responding patients (non-responder = 0.47 ± 0.03, responder = 0.43 ± 0.07, p=0.07) (Figure 30B). Furthermore, the relative reduction in cellular RNF41 expression in erythroids was significantly greater in responding patients compared to non-responders (non-responder = 1.06 ± 0.09, responder = 1.11 ± 0.22, p=0.05) (Figure 30C). These results, if validated in a larger data set, suggest that cellular RNF41 expression level in erythroid precursors may serve as a predictive biomarker for LEN response in MDS. Moreover, the ability of LEN to reduce expression in responding patients may be an important biological marker of therapeutic efficacy. It should be noted that in our in vitro studies, expression of RNF41 decreased after 1hr of LEN exposure, however, in our IHC experiments, RNF41 levels decreased after extended drug exposure. As our IHC RNF41 antibody is reactive to only a small portion of the C-terminus of the protein, it is possible that the reactive site may be masked due to binding of RNF41 to other proteins, such as Jak2 or EpoR, and should be confirmed with an additional antibody.
Figure 30. RNF41 expression decreases in MDS LEN responders. (A) Representative immunohistochemical analysis of MDS bone marrow biopsies. RNF41 is shown in brown and spectrin for erythroid identification is shown in red. The relative expression of RNF41 is increased in LEN non-responders (B) and the relative reduction ratio significantly decreased in LEN responding patients (p=0.05) (C) Furthermore, marked increases of spectrin (red) were observed in responding patients (top IHC panel), whereas there was either no change or a decreases in spectrin staining in non-responders (bottom IHC panels).

Discussion

Our investigations have shown that LEN upregulates the expression of signaling competent Jak2 associated receptor complexes in a concentration-dependent manner, and that EpoR upregulation is a post-transcriptional event yielding accumulation of signaling competent JAK2/EpoR complexes primed to augment Epo response. Inhibition of the E3 ubiquitin ligase activity of RNF41
increases EpoR stability and is responsible for LEN induced upregulation of the receptor, given that forced overexpression of RNF41 inhibits receptor upregulation. Furthermore, cellular expression of RNF41 in bone marrow erythroid precursors in patients who responded to LEN treatment was lower as assessed by immunohistochemistry. Moreover, the relative ratio of RNF41 expression significantly decreased in responding patients compared to non-responders (p=0.05). These data suggest that RNF41 expression in erythroid precursors may be a useful biomarker predictive for response to LEN and merits further investigation in a larger patient cohort.

Our data support the recent findings of LEN inhibition of E3 ligase complexes, including cereblon and MDM2. Collectively, these data suggest that LEN may act as a much broader RING finger E3-ubiquitin ligase inhibitor than originally appreciated. The study of LEN on E3 ligase inhibition should be extended to include more proteins including the other major family of ligases, the HECT ligases, since direct binding sites of the IMiDs have yet to be clearly determined. It is possible that LEN may be inhibiting ligase function via alteration of the ligase complex or binding to the E2 components, and therefore, may not be specific to the RING ligases. Rac GTPases responsible for actin cytoskeletal reorganization and plasma membrane compartmentalization, are activated by IMiDs, however, the mechanism is not known. Perhaps inhibition of specific ligases that ubiquitinate these GTPases underlies the drug activating effects. Furthermore, E3 ligases are important for cellular transport and inhibition by LEN may have a profound effect on the spatial organization of cellular machinery. Additionally, E3 ubiquitin ligases are important for chromatin
remodeling and LEN inhibition on these proteins may have significant effects on gene expression. Recently, the E3 ubiquitin ligase SMURF2 (Smad ubiquitin regulating factor 2) was shown to regulate histone 2B (H2B) ubiquitination, and consequently methylation, through inhibition of the RING finger ligase, RNF20. Perhaps LEN inhibits RNF20 to directly alter expression of erythroid differentiation genes, or in turn, may inhibit SMURF2 effecting RNF20 expression and gene transcription. As these suggestions are speculative, investigations of more ligases (both RING domain and HECT) are warranted, and will provide further insights into the molecular mechanisms of this immunomodulatory agent.

Methods

Reagents and Cells. UT7 cells were grown in alpha-MEM with 20% FBS, 1 % penicillin/streptomycin solution, and 5 ng/ml GM-CSF. HEK-293T cells were maintained in DMEM supplemented with 10% FBS. Normal bone marrow mononuclear cells were purchased from Lonza Walkersville (Walkersville, MD) LEN was purchased from Fisher Scientific (Pittsburgh, PA). Ubiquitin, IL3-R, c-Kit, and CD71 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-Actin antibody was purchased from Sigma Aldrich (St. Louis, MO). EpoR and RNF41 antibodies were purchased from Abcam (Cambridge, MA). Cyclohexamide was purchased from Sigma Aldrich (St. Louis, MO) Bortezomib was purchased from (Sellechchem, Houston, TX).
**Western blotting.** Cells were treated as indicated then harvested and lysed in 1X RIPA buffer with 250µM NaOV₄, 2µg/mL aprotinin, 2µg/mL leupeptin, 0.2µg/mL pepstatin A, and 500µM PMSF. Proteins were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5% dry milk PBST solution (PBS with 0.1% Triton X) and incubated with the indicated antibody. Membranes were washed and developed using ECL or ECL+ according to manufacturer’s protocol.

**Quantitative PCR.** RNA was isolated from UT7 cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) cDNA was generated using High Capcity cDNA Reverse Transcription Kit (Life Technology, Applied Biosystems, Foster City, CA) per protocol. ActB and 18S RNA was used as endogenous controls. EpoR, 18S, and ActB mRNA was detected using TaqMan® Gene Expression Assays (Life Technology, Applied Biosystems, Foster City, CA). Real time PCR was carried out on an ABI PRISM 7900HT Sequence Detection System with triplicate samples using TaqMan® Universal PCR Master Mix with 2 min incubation at 50°C, then activation of AmpliTaq Gold for 10 min at 95°C, then 40 cycles of 15s at 95°C and 1min at 60°C. Data was analyzed using SDS software (v2.3) EpoR mRNA was normalized to endogenous controls.

**Immunoprecipitation.** Two hundred micrograms of protein from total cell lysates were incubated with 2µg of indicated antibody for 2h on ice. Fifty microliters of Protein G Agarose beads (EMD Millipore, Billerica, MA) were added
and incubated overnight on a rotator at 4°C. Bead-lysate slurries were washed 3x in lysis buffer. Sample buffer was then added, and beads were dissociated at 95°C for 5 min. Proteins were separated by SDS-PAGE and immunoblotted with indicated antibodies.

**Immunofluorescence.** BM-MNC were treated with 1µM LEN for 1 hr. The cells were then cytospun for 5 min at 450rpm. Slides were fixed in BD cytofix for 10 minutes at 37°C for 10min, washed with PBS, then blocked in 2%BSA/PBS for 5min at room temperature (RT). Cells were then incubated with primary antibody (1:50 for EpoR and 1:200 for CD71) for 1hr at RT, washed, and incubated in secondary antibody (1:1000) for 1hr at RT. Cells were washed again, DAPI was added, and a cover slip placed on. Micrographs were taken using a Leica TCS SP5 AOBS Laser Scanning Confocal microscope (Leica Microsystems, Germany). Data was analyzed using Image Pro Plus version 6.2 (Media Cybernetics, Inc., Silver Springs, Maryland).

**Transfections.** HEK-293T cells were transfected using the calcium-phosphate method using pMET7/EpoR and pMET7/RNF41 expression vectors kindly provided by Dr. Jan Tavernier from Ghent University (Ghent, Belgium). Briefly, cells were transfected with 2µg DNA by the calcium phosphate method. Three hours after transfection, medium was changed. Cells were either harvested for expression detection or treated after 48hr.
Immunohistochemistry. Paraffin embedded bone marrow core biopsies were deparaffinized using EZ prep solution (Ventana Medical System, Inc, Oro Valley, AZ). Slides were stained sequentially, first with prediluted spectrin (Cell Marque, Rocklin, CA) for 16min followed by secondary for 8min, and was demonstrated with red chromagen. RNF41 secondary antibody (Abcam, ab84409) was added (1:400) for 60 min, with secondary incubation of 16min, and detection by 3,3’ Diaminobenzidine (DAB) chromogen. Retrieval was done with cell conditioning 1 (Ventana Medical Systems, Inc.) Slides were dehydrated and cover-slipped for analysis. Slides were scanned using Aperio™ (Vista, CA) ScanScope XT with a 200x/0.8NA objective lens via tri-liner-array. Three regions from each slide were manually selected by the study pathologist and extracted without compression into Definiens Tissue Studio v3.0 software suite for quantitative analysis. These regional images were segmented using Tissue Composer to classify co-localized regions of interest using the red spectrin staining as the initial nuclear detection marker. The cells of interest were spectrin positive erythroid cells which also displayed RNF41 staining. Therefore, each nucleus within the regions of interest was identified with a hematoxylin threshold of 0.16 and an IHC threshold of 1. Cytoplasms were grown from the nuclei and thresholded into weak, moderate and strong intensity (0.15, 0.28, 0.55, respectively). This complete solution enabled the number of RNF41 positive erythroid cells (colocalized staining) to be identified as well as the mean RNF41 intensity in spectrin positive cells. Segmented based on the intensity of the staining of the various markers, in both nuclear and cytoplasmic areas, where
applicable, and classified as erythroid cells within the region of interest based on the mean intensity of that cellular object. The training algorithm was closely monitored by the study pathologists and applied to all images representative of the patients’ slides.
CHAPTER 5
Discussion

Summary

In the investigations presented here, we have shown that plasma membrane lipid raft microdomains are deficient in MDS, and that EpoR signal fidelity is dependent upon its localization within membrane raft fractions (Chapters 2 and 3). We first showed that Epo stimulation induces raft aggregation and the recruitment of EpoR with the signaling effectors Jak2, STAT5, and Lyn kinase, while CD45, a tyrosine phosphatase negative regulator of the growth factor signal, was sequestered outside of raft fractions. Disruption of lipid rafts abrogated Epo signaling, thereby emphasizing the importance of lipid raft integrity for EpoR signal competence. Our subsequent investigations identified two mechanisms by which LEN can augment EpoR signaling in MDS, 1) through promoting the assembly of lipid rafts and their obligatory signaling constituents, and 2) EpoR up-regulation. We showed that LEN induced the formation of lipid rafts accompanied by recruitment of EpoR, Jak2, and STAT5, while re-partitioning CD45 largely to non-raft fractions independent of ligand engagement (Chapter 3). Induction of raft formation by LEN, and recruitment of EpoR by rhEpo, was dependent upon the activity of Rho and Rac GTPases,
through regulation of F-actin polymerization and cytoskeletal reorganization (Figures 18, 21, and 22). These findings demonstrate the importance of GTPases in LEN’s erythropoietic promoting effects. GTPases are important regulators of hematopoiesis, and their activation by LEN has important consequences, not only on EpoR signal fidelity, but also on erythroid survival as supported by our prior data showing enhancement of colony-forming capacity. F-actin polymerization triggered by the activation of GTPases by LEN is a key effector mechanism regulating lipid raft assembly and aggregation. However, the importance of cytoskeletal reorganization prefacing raft assembly warrants discussion. Expression of DOCK4, a gene integral to cytoskeletal regulation is decreased in MDS compared to normal progenitors, and is associated with decreased F-actin polymerization, increased erythroid fragility, and apoptosis. LEN’s induction of actin cytoskeletal reorganization likely plays an important role in enhancing erythroid viability, in addition to priming progenitors to augment EpoR signal fidelity by inducing the formation of raft signaling platforms, thereby contributing to erythropoietic rescue in responding MDS patients.

The second mechanism by which LEN may augment EpoR signaling is through upregulation of EpoR. We demonstrated that LEN increases EpoR expression through inhibition of the E3 ubiquitin ligase activity of RNF41 (Chapter 4). LEN inhibits both the auto-ubiquitination of RNF41, and RNF41’s ubiquitination of EpoR, thereby increasing receptor stability. Decreased degradation of the Jak2/receptor complex should enhance signaling by
increasing the number of receptors available for membrane translocation and ligand binding. Although erythroid progenitors from MDS patients display comparable levels of surface receptors compared to healthy individuals, lipid rafts are decreased, which compromises EpoR signal competence. Moreover, an increase in RNF41 would promote Type I receptor degradation, which could decrease the number of internalized receptors available for recycling to the plasma membrane after growth factor stimulation, thus decreasing the duration of Epo induced stimulation. Furthermore, we found that erythroid expression levels of RNF41 prior to treatment with LEN in non-del(5q) MDS responding patients was lower than that found in non-responders, whereas the relative reduction ratio after treatment was greater in responders than in non-responders (Figure 30), suggesting greater receptor stability and Epo responsiveness in responding patients. These results also suggest that RNF41 might serve as a potential biomarker predictive for LEN responsiveness in non-del(5q) MDS.

In summary we have shown two mechanisms by which LEN enhances EpoR signaling, 1) through induction of lipid raft assembly accompanied by recruitment of the receptor and signal effectors, and 2) through increased protein expression and stability of EpoR. Collectively, these mechanisms serve to address primary disturbances in membrane raft/cytokine receptor signaling in MDS. LEN promotes lipid raft assembly, rescues ineffective erythropoiesis, and enhances Epo/STAT5 signaling and progenitor survival to correct anemia in LEN-responsive MDS patients. In support of this, the French MDS Group recently reported the results of a randomized clinical trial comparing erythroid
hematologic response to treatment with LEN alone vs. LEN and epoetin beta in transfusion dependent patients with non-del(5q) MDS previously unresponsive to Epo treatment. The results of this study showed that the combined treatment significantly improved erythroid response and frequency of transfusion independence (23% vs. 40%; p = 0.04) (Abstract #7002, 2013 ASCO Annual Meeting, Chicago, IL). Moreover, recent studies implicate allelic deletion or mutation of genes involved in GTPase/cytoskeletal regulation in the pathogenesis of MDS. Examples of this include RhoB, mDia, and Smap1. Smap1 is an ARF6 GTPase-activating protein, and loss of the gene causes a myelodysplastic phenotype in mice, as well as AML development. Loss of either RhoB GTPase or its effector, mDia, similarly causes myelodysplastic phenotypes in mice. These data suggest that novel strategies that promote stimulation of Rho or Rac GTPase activation may address a critical abnormality in cytokine signaling in MDS.

Future Studies

There are a number of additional studies that are warranted to delineate precise disturbances in raft assembly in MDS, GTPase activity and the mechanism(s) by which LEN activates GTPases to effect cytoskeletal changes. First, although we and others have shown that the GTPases induce actin cytoskeleton reorganization resulting in increased raft or synaptosome formation, the specific targets involved in this process are unknown. Identifying key regulatory components in cytoskeletal dynamics could support pharmacologic
strategies for development of novel hematopoietic promoting agents. A summary of possible mechanisms involved in the deregulation of the actin cytoskeleton that may contribute to the pathogenesis of MDS is provided in Figure 31. Currently, there are three GTPases known to regulate the actin cytoskeleton, RhoA, Rac1, and Cdc42. Although RhoA and Rac1 are activated within minutes of LEN treatment, the same response is not observed for Cdc42. In our studies we showed abrogation of LEN induced F-actin polymerization by pretreatment with a Rac1 inhibitor, and recruitment of EpoR into raft fractions was abrogated by both a Rac and ROCK inhibitor, however, the specificity of these inhibitors is insufficient to determine the precise GTPases (or GEFs) involved, or, whether there are any overlapping functions, or compensatory mechanisms. To address this, additional investigations with knockdown of specific proteins are needed. Although there are 20 Rho family GTPases and more than 150 Rac superfamily GTPases, one could begin with the Rac like Rho GTPases (Rac1, 2, and 3) known to be heavily involved in hematopoiesis, and the Rho-like GTPases (RhoA, B, and C), which are known to be involved in cytoskeletal regulation. Furthermore, to confirm that F-actin polymerization is required for lipid raft formation, cells should be treated with a polymerization inhibitor in combination with LEN, however, since F-actin polymerization inhibitors are extremely cytotoxic, caution should be used in interpreting results from such studies.

We speculate that GTPase activation by LEN may occur through E3 ubiquitin ligase inhibition, and therefore, after identification of the specific
Figure 31. Cytoskeletal deregulation in MDS. Deregulation of the actin cytoskeleton may occur via a number of different mechanisms including loss of Rho GTPase activity, deregulation of DOCK4 by hypermethylation, mutation, or deletion, increase in E3 ligase activity resulting in Rho GTPase proteasomal degradation, inhibition of either the Rho GTPases and/or Rho kinase, ROCK, by micro RNAs, loss of the formin family protein, mDia as in del(5q) MDS, or through the Rho regulatory phosphatase, PP2A, which is inhibited by LEN.

GTPases involved in raft formation, targeting ligases responsible for their degradation should follow. At present, there are only three known ligases for Rho family GTPases (SMURF, Cul3, and HACE1). Of these, only Cul3 is a RING domain ligase. Currently, LEN’s ligase inhibitory activity is known to extend only to three RING domain ligases (cereblon, MDM2, and RNF41), however, the effects of LEN on the HECT ligases should also be investigated. At present, there is only one study suggesting a possible IMiD binding site to CRBN (and no data on the binding sites for MDM2 and RNF41, investigations that we are currently pursuing), and that site is at the C-terminus of the protein. Nevertheless, this determination was based solely on site specific point mutations at
amino acids 384 and 386, and decreased inhibition of ligase activity by thalidomide. Direct interactions have yet to be validated, and therefore it is possible that ligase inhibition may not be specific to RING domain ligases. Perhaps, LEN is a broad E3 ligase inhibitor with effects on all ligases. This would particularly hold true for the pseudo-RING/HECT hybrid ligases, which have characteristics of both the RING and HECT ligases. Until the direct binding sites of the IMiDs and E3 ligases are determined, the possibility for broad ligase inhibition should not be discounted. Currently, high throughput E3 ligase screening assays are available, and these may be utilized to determine whether LEN specific ligase inhibition leads to GTPase activation.

Additionally, the question of whether lipid rafts are responsible for the recruitment and aggregation of EpoR and signaling effectors, or, whether these processes are dependent on the receptor itself, merits further investigation. We are currently investigating whether knockdown of EpoR alters LEN’s ability to induce membrane raft assembly to determine if receptor expression is necessary for raft induction. Furthermore, since the cereblon complex was the first identified target of the IMiDs, the role of cereblon should also be investigated to determine its role, if any, in LEN’s promoting effect on raft aggregation and F-actin polymerization. Studies utilizing lentivirus knockdown of cereblon are underway.

Another important follow up study should validate the potential of an erythroid RNF41 score as a biomarker for response to LEN in non-del(5q) MDS. In our preliminary studies, IHC studies were performed on bone marrow biopsy
sections from 6 patients who responded to LEN treatment and 12 non-
responders. Erythroid precursors were identified using the erythroid marker,
spectrin. We found decreased baseline expression of RNF41 in the erythroid
progenitors of responders compared to non-responders that approached
statistical significance (p=0.07). We suspect that by increasing the number of
patients studied, we may obtain statistically significant results. Furthermore, we
found that the magnitude of reduction in erythroid RNF41 expression with LEN
treatment (relative reduction ratio) was greater in responding patients than in
non-responders, further emphasizing the importance of RNF41 in the response
to LEN treatment. LEN’s ability to downregulate RNF41 may be an important
determinant of the compound’s ability to restore effective erythropoiesis. In our
*in vitro* studies, treatment with LEN increased RNF41 protein expression after 1hr
of treatment by inhibiting its autoubiquitination. However, our IHC results
showed that long term LEN treatment in LEN-responsive MDS bone marrow
biopsies resulted in a decrease in expression in erythroid progenitors of
responding patients. The antibody used for IHC recognizes the C-terminus
residues 275-317 on RNF41, whereas the antibody used for western
immunoblots and IP was raised against the full length RNF41, therefore, it is
possible that the decrease in RNF41 after extended LEN exposure was due to
masking of the reactive site. Alternatively extended drug exposure may have
different biological effects that were not explored in our preclinical studies. To
address this, UT7 cells should be treated daily with LEN over an extended period
of time to determine the long term effect of LEN on RNF41 *in vitro*. Additionally,
an alternative RNF41 antibody could be used for IHC staining of bone marrow specimens that recognizes a different binding region and/or reactivity to the full length protein to confirm the observed reduction in expression after long term treatment. Furthermore, we should determine whether EpoR expression increased in erythroid precursors in LEN-responsive MDS patients as a consequence of RNF41 inhibition, and its relationship to changes in RNF41 levels. Lastly, we should determine whether RNF41 expression is increased in MDS patients compared to normal controls, as this may provide insight into disease biology.

Previous studies have shown that plasma membrane raft density is decreased in stimulated neutrophils from MDS patients. In our studies, we show that lipid raft density is also decreased in MDS bone marrow erythroid progenitors, but that lipid raft assembly can be augmented, and all components of the EpoR signaling axis can be aligned within lipid rafts through pharmacologic stimulation of the cytoskeleton. The biological abnormalities underlying the deficiency in raft assembly in MDS warrants further investigation. Although decreased DOCK4 expression represents one mechanism, additional studies should explore whether there is an inherent decrease in Rho GTPase activity in MDS stem and progenitor cells resulting in decreased F-actin polymerization and lipid raft formation. DOCK4 is located at 7q31 and may be regulated through promoter methylation, mutation, or deletion. Interestingly, chromosomal abnormalities at this position are associated with poor prognosis in both MDS and AML. This could account for ineffective cytokine signaling platforms,
decreased EpoR signal fidelity, and decreased erythroid progenitor differentiation and proliferation. As the actin cytoskeleton is closely controlled by the GTPases, a deficiency in GTPase activation in MDS progenitors could result in ineffective erythropoiesis. This notion is supported by finding that MDS neutrophils have decreased Rac activation and decreased F-actin integrity associated with decreased \textit{DOCK4} expression, as well as evidence of myelodysplasia resulting from GTPase and associated protein knockdown.\textsuperscript{206} Alternatively, mDia, the Rho GTPase effector, is another possible deregulated protein of the cytoskeleton, possibly contributing to myelodysplasia.\textsuperscript{142} The mDia gene is located at 5q31.3, the location of the commonly deleted region in del(5q) MDS, and loss of mDia in a mouse model resulted in a myelodysplastic phenotype.\textsuperscript{155,156} Furthermore, it would be of interest to compare lipid raft integrity and aggregation in non-del(5q) MDS LEN responders vs non-responders to determine if raft density or dynamics in response to drug exposure could serve as a biomarker for drug responsiveness. One would expect that lipid rafts may be decreased in LEN responders, and that one mechanism by which therapeutic efficacy is achieved is through rescue of diminished raft density as suggested in Figure 23. Another mechanism by which lipid rafts may be diminished in MDS patients is through defects in raft manufacturing. The processing of sphingolipids and cholesterol with respect to raft manufacturing in MDS patients should be investigated. Defects in either the machinery and/or the translocation of raft components may account for the decreased raft density observed in these patients. This hypothesis is supported by decreased expression of an array of genes involved
in lipid metabolism in MDS patients. Additionally, MDS patients have reported decreased levels of serum cholesterol, low-density lipoproteins (LDL), and high-density lipoproteins (HDL); and the mechanisms responsible for these could similarly be responsible for the deficiencies in raft density. Although, since induction of rafts by LEN occurs within 1hr, there is likely not a defect in cholesterol synthesis suggesting alternative mechanisms. A more likely hypothesis is that there are alterations in the distribution of raft components, and that this is associated with F-actin polymerization and the cytoskeleton. It is well established that in acute myeloid leukemia (AML), there is high cholesterol content in blast cell plasma membranes. This suggests that there may be less available circulating cholesterol available for raft formation and stabilization. Furthermore, a change in F-actin polymerization induced by activation of the GTPases after LEN treatment could quickly redistribute these molecules to create functioning lipid rafts. Another mechanism by which LEN may upregulate the GTPases to induce F-actin polymerization is through inhibition of PP2A. Our laboratory showed that selective cytotoxicity of del(5q) MDS clones by LEN was accomplished through inhibition of the regulatory phosphatases, PP2A and Cdc25c. Rac GTPases, and actin depolymerizing protein, coflin, are known targets of PP2A, inhibition of the phosphatase has been reported to upregulate the GTPases and induce F-actin polymerization. Our laboratory has performed preliminary experiments supporting this hypothesis that are depicted in Figure 32. UT7 cells treated with the PP2A inhibitor, cantharadin, showed an increase in F-actin polymerization determined by immunofluorescence, an
Figure 32. The PP2A inhibitor, cantharadin, induces F-actin polymerization and lipid raft formation. (A) F-actin polymerization is induced by cantharadin. DAPI=blue, F-actin=green. (B) GM-1 dot blot of UT7 cells either stimulated with Epo or treated with cantharadin showing an increase in raft fractions (1 and 2). (C) Western blot of isolated fractions showing increasing Lyn fractionation with GM-1 after cantharadin treatment.

An increase in lipid raft fractionation detected by GM-1 dot blot, and increased Lyn fractionation to the raft domains. However, to validate that PP2A inhibition by LEN induced F-actin polymerization through activation of GTPases, gene transfected cells lines and appropriate controls should be utilized. Lastly, the cytoskeleton is regulated by microRNAs (miRs) (Figure 31).\textsuperscript{216} Both the GTPases and ROCK are regulated through miRs that potentially may be deregulated in MDS. Some miRs known to regulate the Rho GTPases include miR-31, 133, 155, and 185.\textsuperscript{217-220} In fact, miR-155 is identified to be upregulated in MDS CD34+ cells compared to normal counterparts.\textsuperscript{221} Additionally, the miRs-138, 184, and 205 are known down-regulators of the Rho kinase, ROCK.\textsuperscript{222,223}
Investigation of the role of these miRs in the context of MDS and activation of GTPases resulting in cytoskeletal reorganization is warranted. Ultimately, the studies suggested here will provide valuable insight into the disease biology of non-del(5q) MDS, as well as the abnormalities underlying impaired raft assembly in MDS, thereby offering opportunities for development of novel therapeutics for the treatment of patients with MDS.

Implications

The implications of the findings presented here extend not only to the molecular mechanisms of LEN, and the disease biology of MDS, but also to potential biomarkers and novel therapeutic strategies. For example, we propose that RNF41 expression may be used as a biomarker predictive for LEN response for MDS patients. Currently, only about 25% of non-del(5q) MDS patients experience a hematologic response to LEN monotherapy, therefore an accurate biomarker could significantly improve selection of patients most likely to benefit as well as time and cost savings. Furthermore, lipid raft density and raft induction by LEN may be used as markers for response, providing further means for patient selection. Those patients with relatively normal levels of rafts and/or low induction of raft aggregation, may be less likely to respond to LEN compared to those who have decreased levels of raft density. Furthermore, if the mechanism by which rafts are decreased in MDS can be identified, these alterations as well, may provide novel therapeutic targets. These studies would require validation in a prospective treatment trial.
Additionally, small molecules could be used to inhibit the activity of E3 ubiquitin ligases. For example, inhibition of RNF41 would lead to an increase in expression of EpoR and other Type I cytokines, whereas inhibition of the ligases responsible for the degradation of GTPases could induce F-actin polymerization. Specific E3 ligase inhibitors would limit the number of off-target effects that would be expected from using a broad E3 ligase inhibitor. Furthermore, if we could determine alternative methods for activating the GTPases, i.e., by activating GTPases activating proteins (or GAPs), or promoting F-actin polymerization, these methods may be useful in restoring effective erythropoiesis in MDS.

Ultimately, as we gain both disease and drug knowledge, prognosis of patients diagnosed with MDS, and other hematologic malignancies, should prove to be more promising.
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Appendix 1

McGraw, Kathy

From: Basiorka, Ashley A.
Sent: Tuesday, June 11, 2013 1:30 PM
To: McGraw, Kathy
Subject: Permission to use undergraduate thesis

Dear Kathy McGraw:

You have permission to reproduce data from my USF Honors College Undergraduate Thesis from Spring 2012, entitled, “Lenalidomide Upregulates Erythropoietin Protein Receptor Expression Through Inhibition of RNF41 Ubiquitination.”

Sincerely,

Ashley

Please consider the environment before printing this email.