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Regulation of hematopoietic stem cell fate by the ubiquitin proteasome system

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Abstract

Hematopoietic stem cells (HSCs) residing in the bone marrow generate mature blood cells throughout the life of the organism. This is accomplished by careful regulation of HSC activity to balance quiescence, self-renewal and differentiation. Studies of the molecular mechanisms governing HSC maintenance have mostly focused on the role of signaling and transcriptional processes. However, it has recently been demonstrated that protein regulation via the ubiquitin proteasome system (UPS) is crucial for normal HSC function. The loss of which can lead to transformation and leukemogenesis. The effective use of a general and reversible inhibitor of the UPS, Bortezomib, in treating mantle cell lymphoma and multiple myeloma demonstrated that targeting the UPS has therapeutic potential. Thus, understanding the emerging field of how the UPS regulates HSC activity may lead to novel targets for therapy of leukemia.

Hematopoiesis: A balancing act

The generation of mature blood cells throughout the life of an organism is dependent on a rare population of hematopoietic stem cells (HSCs)[1] (see Text Box 1). HSCs possess the ability both to self-renew and differentiate to produce all lineages of blood cells [2–4]. However, HSCs remain mostly quiescent and reside in the G₀ phase of the cell cycle [5]. Maintaining HSC quiescence prevents exhaustion of the stem cell pool and limits the number of replication-associated mutations [4]. The balance between quiescence, self-renewal and differentiation is essential for hematopoiesis and organisms have developed cell extrinsic and intrinsic mechanisms that act in concert to regulate these important fate decisions [6].

Text Box 1

HSC

Hematopoietic stem cells in the adult primarily inhabit the bone marrow. In the mouse, HSCs can be isolated from the mature marker negative or lineage negative (Lin-) compartment. This lineage negative compartment can be further subdivided with cell surface markers c-Kit, Sca-1. HSCs are found within the double positive population or

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LSK subset for Lin⁻, c-Kit⁺, Sca-1⁺. While the LSK subset is enriched for HSCs, it has been mathematically extrapolated that there is only 1 stem cell per 25 cells within the LSK population [76]. Thus to further isolate HSCs the LSK compartment can be further divided using a variety of markers with varying degrees of purity. The LSKF34⁻ population isolates cells that are negative for cell surface markers Flk2, CD34 within the LSK compartment to delineate HSCs. It has been experimentally proven that the LSKF34⁻ population possesses 1 stem cell per 5 cells [77]. Alternatively HSCs can be isolated within the LSK compartment using cell surface markers CD150⁺ and CD48⁻. Using this method, it has been experimentally demonstrated that 1 out of 2 cells is a HSC. Thus this method is often used for isolating a pure stem cell population [78, 79].

In the adult, HSCs reside in the bone marrow within a specialized microenvironment referred to as the stem cell niche [7–9]. The niche is poorly perfused and it is thought that hypoxia is one of the external factors that play a role in HSC maintenance [10]. Growth factors, chemokines, cytokines and adhesion molecules secreted or presented by support cells in the niche are additional extrinsic factors that contribute to HSCs regulation [11]. Complementing external factors, cell intrinsic mechanisms involving cell cycle regulation, cellular signaling, and transcriptional activity all play a key role in processing extrinsic factors and providing an additional level of regulation to maintain the HSC subset [12–19].

Malignancies within the hematopoietic system often arise due to the loss HSC homeostasis. This occurs due to the aberrant function of mechanisms that regulate hematopoietic stem cell fate decisions. While much attention has been given to the various signaling and transcriptional mechanisms regulating HSCs (Reviewed in [6]), little consideration, has been given to the role of ubiquitin proteasome system. This is particularly striking given that the UPS is an important regulator of proteins that are required for both intrinsic and extrinsic mechanisms of HSC maintenance such as Notch [20, 21], c-Myc [13, 17, 19], and Bmi-1 [16, 22] to name a few. Recent work, however, has identified a role for several members of the UPS in control of HSC maintenance. Here we describe this data and discuss how the UPS not only influences HSC homeostasis but also how impaired UPS-mediated regulation of HSCs can lead to transformation.

The UPS: Regulating the balance

The UPS is a complex set of factors that allows the cell to tag proteins for signaling purposes or to target them for destruction. It plays a crucial role in the regulation of a number of cellular functions including cell cycle, protein quality control and transcription [23]. The UPS is also implicated in several developmental processes associated with neurogenesis including progenitor proliferation, specification, migration, and differentiation [24]. Malfunction of the UPS is associated with various disease states, including neurodegenerative diseases as well as hematopoietic malignancies [25, 26].

Ubiquitin modification of proteins occurs via an enzymatic cascade consisting of an ubiquitin-activating enzyme, E1, the ubiquitin-conjugating enzyme, E2, and the ubiquitin-protein ligase E3 which provides substrate specificity (see Text Box 2, Figure 1) [27]. Substrates can be monoubiquitinated or polyubiquitinated. Monoubiquitination is often associated with the alteration of protein activity and/or its location. Polyubiquitination typically leads to irreversible substrate destruction via the 26S proteasome but can also change protein activity (see Text Box 2) [27, 28]. While ubiquitin modification can lead to irreversible proteolysis, the addition of ubiquitin is reversible. Removal of ubiquitin is mediated by a family of deubiquitinating enzymes (DUBs), a majority of which are cysteine proteases [29].

Text Box 2**Ubiquitin conjugation and its consequences**

The ubiquitin modification of proteins occurs via an enzymatic cascade consisting of E1, the ubiquitin-activating enzyme, E2, the ubiquitin-conjugating enzyme and E3, the ubiquitin-protein ligase [80]. Briefly, the E1 enzyme activates ubiquitin in an adenosine triphosphate-dependent manner by forming a thiol ester bond with ubiquitin which is subsequently transferred to E2. The E2 enzyme, also known as ubiquitin-carrier protein, cooperates with the E3 ligase which properly positions the target protein and allows the transfer of activated ubiquitin forming a covalent bond between the carboxy-terminus of ubiquitin and the ϵ -amino group of a lysine residue with the target protein. The two major classes of E3 ligases contain one of two domains: the homologous to E6AP C-terminus (HECT) domain or the RING-finger domain [81]. The HECT family is comprised of large proteins each with the ability to interact directly with E2 ligases and their specific target substrate [82]. In contrast, RING-finger E3 ligases are a complex of proteins that bind the E2 ligase via a catalytic ring finger protein and bind target substrates via a separate member of the complex [83]. Thus, both serve to bring the E2 ligase and the specific target substrate in close proximity in order to facilitate ubiquitin conjugation. Ubiquitin has several acceptor lysines for ubiquitin conjugation which can affect the length and thus function of the ubiquitin modification [27]. Substrates can be monoubiquitinated, or polyubiquitinated. It has been shown that polyubiquitin chains extended from ubiquitin lysine 11 and 48 targets proteins to the 26S proteasome. The 26S proteasome is a large multi-subunit protease complex that is responsible for the proteolytic activity of the UPS and ultimately a protein's irreversible destruction. Monoubiquitination or alternatively polyubiquitin chains extended through lysine 29 and 63 function as a nonproteolytic signal leading to the alteration of protein activity and or location [28]. This is generally associated with the regulation of transcription factors, kinases and chromatin regulators.

As UPS function is crucial to cellular homeostasis, it is not surprising that malfunction of the UPS has been associated with transformation and tumorigenesis (Reviewed in [26, 30, 31]. Although therapeutic targeting the UPS could be predicted as harmful to the cell, the use of Bortezomib (PS-341, Velcade Millennium Pharmaceuticals) for the treatment of mantle cell lymphoma and multiple myeloma, demonstrated that an inhibitor of the proteasome can effectively treat malignancies [32, 33]. Bortezomib functions by blocking proteolytic activity of the UPS. Recently, more specific inhibitors of UPS have been explored with varying degrees of success (reviewed in [34]). The E3 ligase is an ideal therapeutic candidate because it lends substrate specificity to the UPS. However, it has proved a difficult member to target due to the lack of catalytic pockets for small molecule binding [35]. Nevertheless, two recent studies revealed for the first time successful inhibition of an E3 ligase [36, 37]. These data, together with studies showing the efficacy of targeting DUBs in several cancer cell lines [38, 39], demonstrate that targeting the UPS has therapeutic potential for treating various types of cancers. The potential to target the UPS further justifies examining its role in hematopoietic cell fate decisions and hematopoietic malignancies.

Casitas B-cell Lymphoma (c-Cbl), Itch, and Fbw-7: Gatekeepers of quiescence

c-Cbl

The proto-oncogene, c-Cbl, is a RING-finger E3 ubiquitin ligase that is the cellular homolog of v-Cbl, the retroviral transforming gene of the Cas NS-1 murine leukemia virus [40]. c-Cbl is thought to regulate approximately 150 proteins either directly or indirectly [41]. It has a predominantly negative role in regulating the activity of Notch1, c-Kit and STAT5 (an activator of c-myc expression) [42–44]. All of which contribute to HSC maintenance.

Cbl knockout mice exhibit aberrant hematopoiesis [45]. This is demonstrated by a cell autonomous increase in the HSCs without an increase in mature cell output. Functionally, *Cbl*^{−/−} HSCs show enhanced reconstitution capacity in competitive BM transplantation assays and more proliferation in BrdU incorporation experiments. The *Cbl*^{−/−} LSKs also continuously expand in ex vivo culture and are hypersensitive to the cytokine TPO. TPO interacts with its receptor, MPL, leading to JAK2 activation and STAT5 phosphorylation. Phospho-STAT5 translocates to the nucleus where it induces a number of proliferation factors including the oncogene, c-myc [46]. *Cbl*^{−/−} LSKs have increased concentrations of phospho-STAT5 and *Myc* mRNA (encoding cMyc) suggesting *Cbl* deficiency stabilizes “active” STAT5. This data was used to explain the hyperproliferative phenotype observed in these mice, particularly in response to TPO. However, it was not addressed whether overexpression of STAT5 gives a similar result as loss of c-Cbl. Also, because c-Cbl is implicated in regulating over 150 proteins it is possible that other ubiquitination targets of c-Cbl contribute to the phenotype. This work does indicate that the E3 ligase activity of c-Cbl keeps the proliferative capacity of HSCs in check: loss of activity leads to enhanced self-renewal and aberrant proliferation in a normally quiescent adult stem cell compartment.

What are the implications of enhanced proliferation of HSCs in the absence of c-Cbl? Loss of c-Cbl E3 ubiquitin ligase activity in mice eventually led to myeloid proliferative disorders and acute myeloid leukemia [47]. In this work the HSCs were the only population of cells capable of transferring both the pre-leukemic MPD as well as the leukemic phenotype to irradiated recipient mice. This demonstrates that the biochemical changes in the HSC subset due to the loss of c-CBL ligase activity can lead to malignant hematopoiesis. In humans, *CBL* is mutated in 5–15% of myeloid proliferative disorders, although whether this is causal is unclear [48–52].

Itch

Itch belongs to the HECT family of E3 ligases. It is proposed to have more than 20 cellular targets including Notch [30]. In *Itch*^{−/−} mice the absolute number of HSCs is increased without an increase of mature cells [53]. Competitive bone marrow transplantation experiments show enhanced self-renewal capacity of *Itch*^{−/−} HSCs, and these cells were also more proliferative in BrdU labeling experiments. Under the physiological stress of myeloablation via 5-fluoruracil (5-FU) *Itch*^{−/−} cells showed an enhanced hematopoietic recovery. This was demonstrated by increased red blood cell and platelet count as well as increased survival of *Itch*^{−/−} mice after sequential 5-FU treatment. Additionally, *Itch*^{−/−} cells also had sustained progenitor potential assessed by colony forming assays and in vitro culture followed by bone marrow transplantation. Knockdown of Notch, a known target of Itch, in Itch-deficient progenitors demonstrated a partial rescue suggesting that the *Itch*^{−/−} phenotype may in part result from increased stability of active intracellular Notch (ICN). It therefore appears that Itch, similar to c-Cbl, functions as a negative regulator of stem cell self-renewal and proliferation via a known regulator of hematopoiesis, in this case Notch.

Also similar to the c-Cbl work, the possibility that modification of another Itch target plays a role in causing the observed HSC defect was not ruled out.

Fbw-7

Fbw-7 is a RING finger E3 ligase that plays a role in regulation of approximately 20 proteins including c-myc and Notch. Germline deletion of *Fbw7* results in embryonic lethality around E10.5 due to vascular defects, which are attributed to the stabilization of Notch4 in the embryo [54, 55]. Conditional deletion of *Fbw7* (referred to here as *Fbw7*^{-/-}) in the hematopoietic system lead to an increase in the frequency of actively cycling LSKs, with eventual loss of the HSCs [20, 21]. *Fbw7*^{-/-} HSCs were unable to compete in competitive bone marrow reconstitution assays demonstrating that HSC defects were cell autonomous [20, 21]. Furthermore, *Fbw7*^{-/-} LSK cells down regulate genes involved in HSC quiescence implying a global loss of quiescence characteristics [21]. This is most likely due to change of transcriptional activity or stability of one or more transcription factors that are targets of Fbw7. In fact, the defects in the HSC compartment in *Fbw7*^{-/-} animals could be overcome by reducing concentrations of c-myc protein, a known Fbw7 target, using combined *Fbw7*^{-/-} *Myc*^{+/-} deficient mice. This indicates that the reduced frequency and eventual loss of the HSCs resulted from stabilization of the oncogene, c-Myc [17, 20, 21]. While these results exclude several Fbw7 targets, one cannot conclusively attribute the phenotype to one substrate as additional Fbw7 targets that were not examined may still play a role in the phenotype. Similar to *c-Cbl*^{-/-} and *Itch*^{-/-} mice, Fbw7-deficient mice have an increase in cycling progenitor cells. While this leads to enhanced stem cell activity in transplantation experiments for both c-Cbl- and Itch-deficient cells, Fbw7-deficient cells are less fit and are lost in transplantation experiments. This suggests that while targets for E3 ligases may overlap, their activity within the same cells is unique and specific.

Although a significant percentage of Fbw7 null mice develop anemia as a result of HSC defects *Fbw7*^{-/-} animals can also develop lymphoblastic thymic lymphoma and T cell acute lymphoblastic leukemia (T-ALL) later in life [20, 56]. Like in the HSC compartment, failure to exit the cell cycle in the thymic lymphoma might directly result from abnormal c-Myc protein stability. In contrast, in mice that develop aggressive T-ALL aberrant Notch stabilization drives the leukemic phenotype [45].

VHL, Usp1 and MDM-2 : Surviving differentiation

VHL

The von Hippel-Lindau protein (VHL) is a substrate recognition protein of a RING-finger E3 ligase. Its best characterized substrate is hypoxia-inducible factor-1 α (HIF-1 α) [57]. HIF-1 α is a transcription factor important for ~100–200 genes that promote survival under hypoxic conditions. Under normal O₂ conditions HIF-1 α is recognized by VHL leading to HIF-1 α degradation [58].

The loss of one or two alleles of VHL stabilized the HIF-1 α protein and induced HSC quiescence in a HIF-1 α dose-dependent manner [59]. Induced quiescence was determined by an increase of LSK number as well as an increase of LSKs that were in G₀ of the cell cycle as measured by Ki67 as well as Pyronin Y. *Vhl*^{-/-} mice also possessed an attenuated differentiation status of their peripheral blood. Reflecting the inactivity in the *Vhl*^{-/-} LSK population, these cells were unable to compete against wildtype LSKs in competitive transplants due to a defect in homing as well as an increase in apoptosis within the transplanted population. As previously mentioned the HSC niche is hypoxic and this environment is thought to maintain the HSCs in a mostly quiescent state. The Vhl-deficient mice work shows that HSCs express high amounts of HIF-1 α necessary to survive in such

an environment. It also demonstrate how HIF-1 α needs to be degraded by VHL as HSCs move out of this hypoxic environment in order to proceed with proliferation and differentiation key to normal hematopoiesis.

Usp1

Ubiquitin specific protease USP1 is cysteine protease DUB that deubiquitinates FANCD2 and thus is a regulator of the Faconia anemia (FA) pathway [60]. During DNA damage or S-phase monoubiquitinated FANCD2 is targeted to chromatin, where it interacts with FA proteins and assembles in nuclear foci [60]. The FA pathway mediates DNA repair and promotes resistance to DNA crosslinking [61]. Patients deficient for one of these 13 genes have chromosome instability syndrome characterized by childhood onset aplastic anemia, congenital anomalies, and heightened cancer susceptibility. Bone marrow failure in FA children is attributed to excessive apoptosis and subsequent failure of the HSC compartment [62]. Although *USP1* is not one of the 13 genes mentioned because no mutations in *USP1* have been identified in FA patients, *Usp1*^{-/-} mice have similar characteristics to FA gene KO mice (e.g. small size, infertility, chromosome instability) [63]. Loss of *Usp1* in HSCs leads to increased numbers of LSKs, a population enriched for HSCs [64]. These LSKs are unable to compete in BMT and exhibited reduced CFU ability. However, the reason for this defect is unclear viability, cell cycle and homing experiments were not performed. In *Usp1*^{-/-} mice both ubiquitinated FANCD2 and ubiquitinated PCNA2 (another *Usp1* target) increase. Thus USP1, in part due to its regulation of FANCD2, it is able to protect HSCs from DNA damage during divisions that are necessary to maintain the stem cell pool.

MDM-2

The hypoxic niche leads to low concentrations of reactive oxygen species (ROS) in HSCs: ROS increase as proliferation and differentiation move out of the niche. ROS is a DNA damage signal that leads to the activation of p53 [65, 66]. MDM-2 is a RING finger E3 ligase that targets p53 for degradation as well as represses the accumulation of p53 by binding to its N-terminus inhibiting its transcriptional activity [67]. *Mdm2* knockout mice are embryonic lethal. However, the loss of p53 rescues this lethality [68, 69]. Several studies using hypomorphic alleles of *p53* and tissue specific deletion of MDM-2 have allowed a better characterization of MDM-2 deficiency [70]. The MDM-2/p53 pathway is a well characterized intricate pathway that has been reviewed elsewhere [71] [72] and due to space limitations will only be discussed in terms of HSCs.

Abbas et al analyzed *Mdm-2* deficient mice crossed to a *p53* hypomorphic allele (*p53*^{515C}) [73, 74]. These *p53* hypomorphs possessed cell cycle arrest capability but lost the ability to induce apoptosis [75]. This rescues the *Mdm-2*^{-/-} lethality but these mice die postnatally at day 13 due to hematopoietic failure. This work demonstrated that the loss of MDM-2 activity led to a stabilized p53, which impeded hematopoiesis via induction of cell cycle arrest, senescence and ultimately cell death in HSCs and progenitors. This was a cell intrinsic defect which was completely dependent on p53 and alleviated by treatment with antioxidants, demonstrating the necessity of ROS to induce p53. This work highlights the MDM-2/p53 pathway in hematopoiesis. It indicates MDM-2 is required to regulate p53 levels as ROS levels increase during hematopoiesis. This allows stem cell survival and continuation of differentiation.

Concluding Remarks

In the 40 years since its identification, the UPS has emerged as an important regulator of protein stability and activity. Recently this has extended to a role for UPS in HSC maintenance and differentiation. In order to maintain normal adult hematopoiesis HSCs

must continuously make fate decisions regarding quiescence, self-renewal and differentiation. Members of the UPS summarized here (Table 1, Figure 2) play a crucial role in these processes and thus the UPS is a master regulator of HSC maintenance.

Much remains to be learned about how UPS regulates HSC homeostasis in terms of mechanism. From much of the work discussed here, it is difficult to assign a clear and unequivocal target to explain the effects of aberrant UPS activity. This is an inherent problem with studying the UPS given its capacity to modify hundreds of proteins. It may be the case that the observed phenotype is due to a combined effect of several target substrates and not just one. Additionally, ubiquitin modification can change protein activity, and UPS targets that change in activity rather than stability are more difficult to identify. Furthermore, assessing protein activity and stability often requires large quantities of cells making analysis of rare HSCs cumbersome. Nevertheless, the role of the UPS in HSC maintenance remains a burgeoning field. Future questions of particular interest are whether individual members can be effectively targeted therapeutically and what role regulators of the UPS play in HSC development?

The ability of the UPS to regulate HSC biology suggests that it is also an influential player in leukemogenesis. An increasing number of studies summarized here, show that mutation in or loss of UPS members leads to leukemia. Understanding the mechanisms that regulate the UPS and how these mechanisms can, in turn, be targeted in various types of malignancies offers an exciting new front in understanding normal and malignant hematopoiesis.

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Figure 1. Ubiquitin proteasome members mediate HSC maintenance

The UPS plays a role in HSC homeostasis and is involved in several aspects of HSC cell fate. HSCs reside in a hypoxic niche where they receive both extrinsic and intrinsic signals keeping them in a generally quiescent state. As HSCs proliferate and become more lineage committed, they move out of their niche into environments where reactive oxygen species (ROS) concentrations increase. Thrombopoietin (TPO) found in the bone marrow hematopoietic stem cell niche activates STAT5 leading to proliferation by activation of c-myc. c-Cbl functions to regulate the amount of Stat5 in HSCs thus limiting proliferation [45]. HSCs express high amounts of Itch which restricts active Notch signaling keeping HSCs undifferentiated [53]. Fbw-7 is also highly expressed in HSCs and directly limits of the amount of c-myc protein, thus affecting the proliferative capacity of HSC and maintaining quiescence [17]. In order to survive in this hypoxic environment HIF-1 α is highly expressed. The movement out of this hypoxic environment necessitates the degradation of HIF-1 α which is mediated by VHL [59]. The increase of ROS is a DNA damage signal which leads to the activation of p53. The levels of p53 are regulated by MDM-2 allowing the cells to survive differentiation [75]. USP1, in part due to its regulation of FANCD2, it is able to protect HSCs from DNA damage during divisions that are necessary to maintain the stem cell pool [64].

Figure 2.

Table 1

Summary of ubiquitin proteasome system members influential in hematopoiesis

Gene	Substrate	Loss of function phenotype	References
<i>c-Cbl</i>	STAT5	Stem cell hyperproliferation, enhanced self-renewal, no stem cell exhaustion, myeloid leukemia	45–49
<i>Itch</i>	NOTCH	Stem cell hyperproliferation, enhanced self-renewal, no stem cell exhaustion	53
<i>Fbw-7</i>	c-MYC	Stem cell hyperproliferation, T-ALL	21; 17
<i>Usp1</i>	FANCD2	Increase LSK compartment, reduced self-renewal	64
<i>Mdm-2</i>	P53	Hematopoietic failure, Lethal day 13 postnatal	75
<i>Vhl</i>	HIF-1a	Increased quiescence, attenuated differentiation, reduced viability, reduced homing	59