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Review

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Regulation of stem cell function by protein ubiquitylation

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Abstract

Tissue homeostasis depends largely on the ability to replenish impaired or aged cells. Thus, tissue-resident stem cells need to provide functional progeny throughout the lifetime of an organism. Significant work in the past years has characterized how stem cells integrate signals from their environment to shape regulatory transcriptional networks and chromatin-regulating factors that control stem cell differentiation or maintenance. There is increasing interest in how post-translational modifications, and specifically ubiquitylation, control these crucial decisions. Ubiquitylation modulates the stability and function of important factors that regulate key processes in stem cell behavior. In this review, we analyze the role of ubiquitylation in embryonic stem cells and different adult multipotent stem cell systems and discuss the underlying mechanisms that control the balance between quiescence, self-renewal, and differentiation. We also discuss deregulated processes of ubiquitin-mediated protein degradation that lead to the development of tumor-initiating cells.

Keywords differentiation; malignancy; proteasome; stem cells; ubiquitin
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See the Glossary for abbreviations used in this article.

Stem cells: concepts and definitions

Embryonic stem cells and adult tissue-resident stem cells are of great interest in biology and medicine due to their unique characteristics [1]. They have the ability to self-renew, which is defined as the capacity to proliferate while being able to differentiate to downstream cellular types upon proper stimuli from their environment. This unparalleled cellular plasticity of stem cells identifies them as key determinants of tissue equilibrium.

Although progenitor cells also have the ability to self-renew, this is usually a short-term characteristic [2]. The long-term self-renewal capacity of stem cells is essential to supply tissues with differentiated progeny throughout the life of the organism. Adult stem cells reside in specialized microenvironments called niches and manifest different degrees of quiescence, depending on the specific organ characteristics. For example, hematopoietic stem cells (HSCs) are dormant [3], whereas mammary stem cells (MaSCs) appear to be cycling [4] and intestinal stem cells (ISCs) proliferate rapidly [5].

Stem cells can divide symmetrically or asymmetrically [6; Sidebar A]. Symmetric cell divisions ensure that all elements are distributed equally between the two identical daughter stem cells, and differentiation—usually of only one of the daughter cells—occurs at a later stage. Asymmetric cell divisions, on the other hand, lead to the unequal division of stem cell components in a process that involves proper positioning of the mitotic spindle [7]. As a result, one cell remains a stem cell, whereas the other adopts a different cell fate. Asymmetric divisions also physically displace one daughter cell from its relative position to the niche, leading to its differentiation.

Signals from the niche microenvironment are critical in regulating intrinsic stem cell transcriptional programs. Various signaling pathways such as Wnt, Hedgehog, Notch, TGF-β/BMP, and JAK/STAT act in concert to shape the regulatory networks that control cell cycle progression or exit, differentiation, and homeostasis. Disturbing the balance between these signaling pathways can deregulate these processes and lead to tumor formation [8]. Thus, the precise control of these pathways, both in stem and in niche cells, is crucial to execute proper developmental programs. The control of protein stability and/or activity by ubiquitylation is essential in the control of the above-mentioned signaling pathways, and its manipulation can either support or alter stem cell properties.

The nuts and bolts of ubiquitylation

The regulation of protein stability is a crucial function in the control of cell plasticity. The ubiquitin-proteasome system (UPS) is a fundamental mechanism to regulate protein stability, quality control, and abundance. Ubiquitylation is a post-translational modification process that results in the covalent conjugation of the small, highly conserved, 76-amino acid protein ubiquitin to lysine residues of substrate proteins through a cascade of enzymatic reactions [9]. These events involve the activation of ubiquitin using ATP by

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Glossary

APC	adenomatous polyposis coli
BMP	bone morphogenetic protein
CKI	casein kinase 1
CRL	Cullin-RING-ligase
Dnmt1	DNA methyltransferase 1
DUB	deubiquitylating enzyme
FANCD2	Fanconi anemia group D2 protein
Fbxw7	F-box/WD repeat containing protein 7
GSK3β	glycogen synthase kinase 3 beta
HECT	homologous to E3-AP C-terminus family of E3 ligases
HIF-1a	hypoxia-inducible factor 1, alpha subunit
iPS	induced pluripotent stem cell
JAK	Janus kinase
Lgr5	leucine-rich repeat containing G-protein-coupled receptor 5
LSK	lineage-negative Sca1-positive cKit-positive cells containing the hematopoietic population
Mbi1	mind-bomb 1
Mdm2	mouse double minute 2 homolog
Psmd	proteasome 26S subunit, non-ATPase
REST	RE1-silencing transcription factor
RING	really interesting new gene family of E3 ligases
ROS	reactive oxygen species
SILAC	stable isotope labeling by amino acids in culture
Skp2	S-phase kinase-associated protein 2
Smad	small body size/mothers against decapentaplegic; TGF-β signaling transcription factors
STAT	signal transducer and activator of transcription
T-ALL	T-cell acute lymphoblastic leukemia
TGF-β	transforming growth factor beta
UPS	ubiquitin-proteasome system
USP1	ubiquitin-specific peptidase 1
VHL	Von Hippel-Lindau
Wnt	homologues of the *Drosophila* "wingless" signaling proteins

E1-activating enzymes, followed by its transfer to E2-conjugating enzymes and finally the formation of an isopeptide bond between ubiquitin and the substrate protein catalyzed by E3 ligases, which confer substrate specificity [10]. This cascade can be repeated multiple times resulting in polyubiquitylated substrates, where each ubiquitin moiety is conjugated to the previous one.

Ubiquitin contains seven lysines (K6, K11, K27, K29, K33, K48, and K63), all of which can be acceptors for the next ubiquitin, as can the amino-terminal methionine. As a result, polyubiquitylation can generate substrates tagged with different types of ubiquitin chain, as well as branches of mixed-chain composition [11]. These different chain linkages result in different degrees of polyubiquitylated chain compaction, which can mediate diverse cellular outcomes. For example K11-linked chains, which have some degree of structural flexibility, have been implicated in mitotic degradation [12], whereas K63 chains, which have open, linear-like conformations, have been associated with the activation of kinases [13, 14]. A well-studied type is the highly compact K48-linked ubiquitin chain, which serves as the canonical signal for degradation by the proteasome [15]. Monoubiquitylation and polyubiquitylations have been implicated in regulating virtually all cellular signaling pathways and processes [16], in addition to maintaining proteostasis. The different ubiquitin chains are recognized by ubiquitin-binding domains of "reader" proteins, thereby deciphering this three-dimensional code [17, 18].

The proteasome is a multimeric enzymatic complex that consists of the 20S catalytic core and one of three regulatory particles, 19S, 11S/PA28, or Blm10/PA200. The core contains the catalytic sites for degradation, and the regulatory particle is responsible for substrate recognition, removal of the polyubiquitin chains, unfolding and translocation into the catalytic cavity [19]. Regulatory particles contain ubiquitin-binding receptors [20], deubiquitylating enzymes (DUBs) [21], and ATPases [22] in order to perform these functions. The catalytic core has trypsin-, chymotrypsin-, and caspase-like proteolytic activities and degrades proteins in a processive manner, generating short peptides. The assembly and structure of the proteasome are dynamically controlled to enable the degradation of a wide variety of substrates and thereby regulate multiple cellular functions [19, 23, 24].

Ubiquitylation is often deregulated in many types of disease, including cancer, neurodegenerative, and immune disorders. For this reason, it is the focus of intense research that aims to develop effective inhibitors of UPS activity that could selectively kill altered cells. Bortezomib, also known as Velcade, is one of the best-characterized proteasome inhibitors and has been used to treat multiple myeloma, as well as a number of solid tumors [25, 26]. Similarly, other proteasome subunits are current targets for drug development [27].

E3 ubiquitin ligase enzymes are responsible for the recognition of substrates for ubiquitylation. In humans, there are more than 600 E3 ligases, divided into three major families, the HECT, RING, and RING-between-RING (RBR) ligases [10, 28–31]. Although they are highly selective enzymes, substrate recognition and ubiquitylation often depend on the cross-talk between different post-translational modifications. A well-characterized example is phosphorylation-dependent ubiquitylation, when phosphorylation is a direct pre-requisite for substrate recognition by the ubiquitylation machinery [32]. However, phosphorylation can also block recognition of substrates, suggesting that phosphatases can also regulate this process [33].

Ubiquitylation of substrates followed by proteolytic degradation is a unidirectional process that involves the physical unfolding and cleavage of a protein. However, prior to being processed by the proteasome, ubiquitin removal can be catalyzed by DUBs, preventing proteasomal cleavage and resulting in protein stability. Five families of DUBs are known to exist, the majority of which are cysteine peptidases. DUBs are emerging as important players in development and the identification of a growing number of substrates has been the focus of much attention in a wide variety of systems. DUBs are implicated in chromatin regulation, transcriptional control, and the modulation of mitogenic pathways. Their function is often deregulated in malignancies, leading to a stabilization of oncogenic or anti-apoptotic factors [34, 35].

In all, ubiquitylation and protein degradation by the proteasome constitutes a highly regulated and evolutionarily conserved process that affects development and tissue physiology, and is frequently deregulated in disease. In the biology of stem cells, ubiquitylation plays key roles in self-renewal and cell fate specification. It provides an additional layer of stem cell regulation at the post-translational level and extends to multiple cellular processes, including the modulation of extracellular matrix composition, surface receptor trafficking and signaling, control of the cell cycle, transcription factor abundance, and deposition of epigenetic marks. Here, we

discuss how ubiquitylation mediates the balance of cell fate decisions in different stem cell systems.

Ubiquitylation pathways in embryonic stem cells

Embryonic stem cells (ESCs) are derived from the inner cell mass of pre-implantation blastocysts [36, 37]. They are pluripotent and have remarkable cellular plasticity, as they can differentiate to all somatic and germ cell lineages of the embryo proper. ESCs can be cultured *in vitro* maintaining their unique characteristics indefinitely. Additionally, they can differentiate to various cell types under proper culture conditions. These unique properties of ESCs—including their rapid proliferation—make them exceptionally valuable to study mechanisms that dictate protein stability. Ubiquitylation pathways can modulate ESC functions on multiple levels, and thus, there is increasing interest in their use for the discovery of drugs that alter protein abundance.

At the level of transcription, a core factor circuitry consisting of Oct4, Sox2, Nanog, and c-Myc regulates the balance between ESC self-renewal and differentiation. These proteins directly control the levels of each other, creating a positive feedback loop that sustains high levels of expression [38]. Furthermore, they can form complex networks with multiple transcription and chromatin-regulating factors. The dynamic interactions among these components ultimately regulate differentiation and lineage specification processes [39]. Changes in transcription factor abundance can result in the tipping of this balance toward specific patterns of differentiation. As a result, regulation at the post-translational level by ubiquitylation is crucial and can specify these processes.

There are several examples of ubiquitin-modulating enzymes that control transcription factor abundance in ESCs. The HECT family E3 ligase Wwp2 has been suggested to ubiquitylate Oct4 in both mouse and human ESCs [40, 41], decreasing Oct4 transcriptional activity and leading to its proteasomal degradation (Fig 1A). In the same studies, Wwp2 was also proposed to undergo auto-ubiquitylation, providing an additional level of complexity [42]. Although there are a lot of open questions regarding this function *in vivo*, as well as how ubiquitylated Oct4 interacts with additional ESC components, these studies suggest that ubiquitylation fine-tunes self-renewal by controlling Oct4 levels and activity. As expression levels of Wwp2 correlate with pluripotency, it would be interesting to investigate how these functions control cellular reprogramming, given that Oct4 is a crucial factor in iPS generation [43].

Another example of transcription factor modulation in ESCs is c-Myc ubiquitylation by SCF^{Fbxw7} [44] (Fig 1A). This interplay illustrates how ubiquitylation orchestrates ESC differentiation. c-Myc is primed for ubiquitylation by Gsk3-dependent phosphorylation on Thr58, creating a phosphodegron that promotes its proteasomal degradation [45]. Loss of c-Myc protein, a key transcriptional determinant of ESC function, induces an irreversible transition toward differentiation. Furthermore, degradation of c-Myc by SCF^{Fbxw7} is involved in cellular reprogramming, as mouse embryo fibroblasts in which Fbxw7 is depleted are able to reprogram to iPS cells more efficiently [44]. These results demonstrate the importance of dynamic ubiquitylation events on the establishment of cell fate specification.

Similar to Oct4 and c-Myc, the levels of Nanog are also regulated by ubiquitylation [46] (Fig 1A), and this process has been linked to phosphorylation [47]. Nanog is known to be a phosphoprotein in mouse ESC [48], and recent efforts in human ESCs have tried to

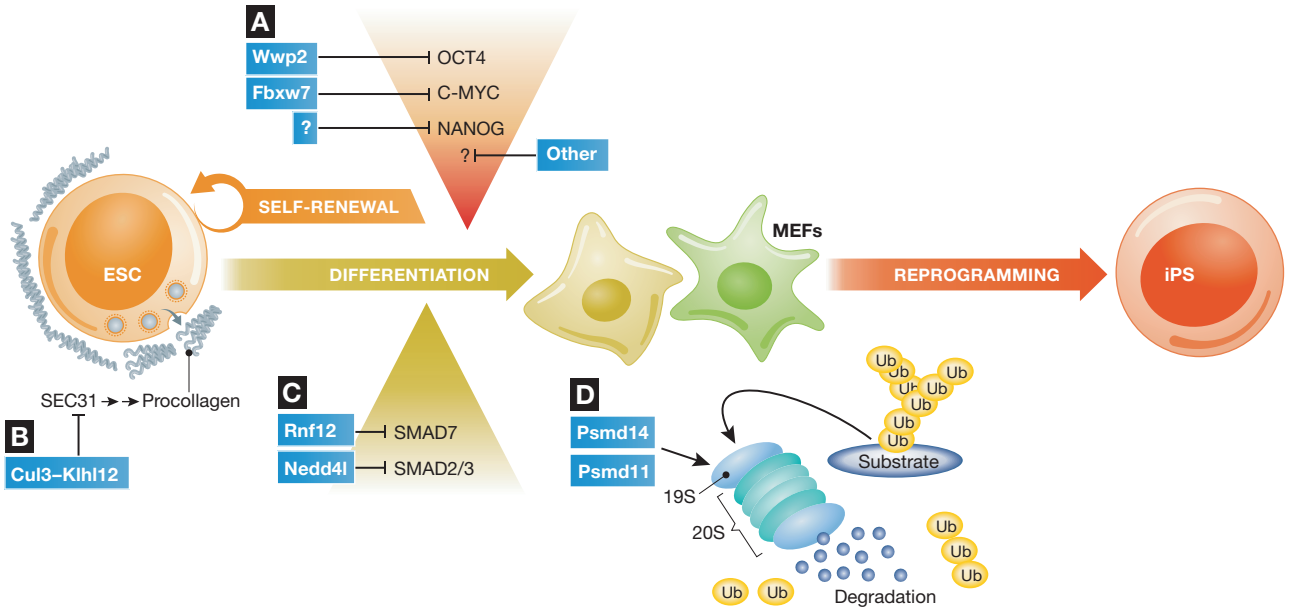

Figure 1. Ubiquitylation regulates ESC pluripotency, differentiation and iPS cell generation.
(A) The E3 ligases SCF^{Fbxw7} and Wwp2 regulate core transcription factor—such as c-Myc and Oct4—abundance and functions in ESCs. Additional enzyme–substrate pairs may control transcriptional regulation of ESCs. SCF^{Fbxw7} controls cellular reprogramming and iPS generation, in addition to differentiation, through c-Myc stabilization. (B) Cul3-Klhl12 ubiquitylates Sec31, regulating COPII vesicle size and procollagen export to the extracellular matrix. (C) Ubiquitylation regulates signaling components in ESCs. Nedd4l and Rnf12 regulate Smad2/3 and Smad7 levels, respectively. (D) Enzymatic functions of the proteasome control self-renewal and differentiation of ESCs, as well as cellular reprogramming. Psmd14 and Psmd11 regulate 19S regulatory particle activity and assembly, respectively.

understand this post-translational cross-talk. Phosphorylation of Nanog alters its degradation rate, resulting in an increased stability in human ESCs [47]. Its ubiquitylation and proteasomal degradation depend on specific sequence motifs, deletion of which has dramatic effects on Nanog stabilization [46]. Although the E3 ligase responsible for this activity remains elusive, the maintenance of Nanog levels has a big impact on ESCs, as elevated *Nanog* expression sustains LIF-independent self-renewal [49]. Therefore, it is intriguing to speculate that regulation at the post-translational level by ubiquitylation can have similar effects. However, additional work needs to clarify previous efforts to this direction. Interestingly, stemness proteins, such as Nanog, have been linked to malignancies and correlate with tumor progression and poor prognosis [50, 51]. As the enzymes implicated in the post-translational control of Nanog levels have also been associated with pathology [52], a tempting possibility is that the ubiquitylation and proteasomal degradation of key pluripotency factors is not only a determinant of ESC differentiation but can also determine malignant transformation and disease progression.

In addition to level of transcription factors, ubiquitylation can regulate other aspects of ESC function, such as receptor signaling and vesicle trafficking. Ubiquitylation mechanisms have been elegantly linked to secretion and support of the extracellular matrix [53]. The ubiquitin ligase Cul3—through its adaptor Klhl12—was found to monoubiquitylate Sec31, a basic constituent of COPII vesicles in ESCs (Fig 1B). This ubiquitylation controls the size of COPII vesicles, enabling the export of large vesicle cargo, such as procollagen fibers. These groundbreaking findings demonstrate that ubiquitin pathways control not only intracellular constituents, but also the extracellular space, expanding the perception of how ubiquitylation regulates integrin signaling and cell division in early embryo development.

Ubiquitylation also impacts signal transduction in ESCs, shaping their differentiation potential toward specified lineages. Components of the TGF-β signaling pathway, for example, are well-characterized targets of ubiquitylation. The E3 ligase Nedd4L can tag phosphorylated Smad2/3 for degradation in ESCs [54], skewing differentiation toward mesodermal lineages (Fig 1C). On the other hand, the inhibitory Smad7 was recently suggested to be a target of Rnf12 [55]. ESCs deficient of *Rnf12* are unable to induce the formation of anterior mesoderm or inhibit neuronal differentiation when challenged with Activin and BMP/Smad, respectively.

Collectively, the above examples outline the diverse and complementary nature of ubiquitin-mediated modifications in ESCs. Relative protein abundance, vesicle trafficking and signal transduction, all regulated by ubiquitylation, control important cell fate decisions. Thus, the functional role of ubiquitin extends to fundamental stem cell processes, emphasizing its importance in development.

Finally, we would like to focus on the role of the proteasome in stem cells, as a machinery that balances opposing developmental processes. Interestingly, the various regulatory particles have different effects on proteasomal activity. As a result, their functions are distinct in ESCs. For instance, the 11S/PA28 activator is required to eliminate oxidatively damaged proteins during differentiation [56, 57]. On the other hand, the 19S regulatory particle is essential for maintenance of self-renewal and turnover of ubiquitylated proteins. In mouse ESCs, the deubiquitylating enzyme Psmd14, an integral subunit of the 19S particle, is necessary for proper self-renewal [44] (Fig 1D). Loss of Psmd14 leads to differentiation with the simultaneous accumulation of polyubiquitylated proteins, which can be fully rescued by the restoration of this enzymatic activity. Furthermore, Psmd14 is required for iPS cell generation, further emphasizing the importance of proteasome function in pluripotency (Fig 1D). In human ESCs, PSMD11—which is a component of the proteasome lid—was shown to play an instrumental role in the assembly of the 20S core with the 19S particle, thus regulating proteasome activity [58]. Decreased levels of this subunit lead to diminished cleavage activity and differentiation. Additional work is certainly needed in order to clarify the exact changes in proteasome architecture and identify the specific effects on stem cell elements that trigger differentiation. However, the above examples demonstrate the regulatory roles that proteostasis, and therefore the proteasome subunits, have on stem cell biology. In agreement with this, proteasomes have been suggested to restrict permissive transcription by degrading preinitiation complexes at loci important for development, thereby preventing differentiation [59]. Conversely, transcription factors such as OCT4 have been shown to directly or indirectly control the expression of multiple proteasome subunits in an interesting feedback loop [60]. In summary, the work discussed above demonstrates the diversity and importance of ubiquitylation functions in ESC regulation.

Ubiquitylation pathways in adult stem cells

The regenerative potential of tissues relies on specialized subsets of multipotent stem cells that can give rise to all the cells that make up a given tissue. Those tissue-specific stem cells maintain a tightly controlled balance between quiescence, self-renewal, and differentiation. However, many of the molecular mechanisms governing stem cell fates are poorly understood. Some of them are common for several types of stem cells, whereas others are tissue specific. The ubiquitin system has an important role in the regulation of fundamental cellular functions such as cell cycle, DNA damage repair, protein quality control, and transcription; however, little is known about its impact in regulating adult stem cell differentiation and lineage specification. In the last years, the development of several E3 ligase-knockout mouse models has revealed important functions of the ubiquitin system in adult stem cell biology.

Hematopoietic stem cells as a paradigm of regulation by ubiquitin

Hematopoietic stem cells have the capacity to regenerate the blood cell lineage throughout the life of an organism [61–63]. This capacity depends on their remarkable self-renewal and differentiation properties. Most of the HSCs remain quiescent in specialized niches of the bone marrow. Only in response to specific stimuli, HSCs can re-enter the cell cycle and self-renew or differentiate [64]. Quiescence and self-renewal prevents exhaustion of the HSC, whereas differentiation induces the production of the different cell lineages. Therefore, the molecular mechanisms controlling the balance between quiescence, self-renewal, and differentiation are tightly regulated. Improper control of this equilibrium may result in hematopoietic failure or cancer [65]. The regulation of these cellular events is achieved on two different levels. First, in the HSC niche, growth factors, chemokines, cytokines, and other secreted

molecules constitute cell-extrinsic networks controlling HSC maintenance [66]. Second, complex cell-intrinsic signaling pathways involving cell cycle, proliferation, growth, and survival have been shown to be essential in HSC homeostasis [65, 67–73]. The UPS plays an important role in regulating protein functions required in both networks. Ubiquitylation of Notch [74] and c-Myc [68, 71, 72, 75, 76] are among the most characteristic examples of such regulation. Moreover, impaired ubiquitin-mediated regulation of HSCs has been linked to malignant transformation events [76].

E3 ligases controlling HSC quiescence Loss of HSC quiescence leads to hematopoietic exhaustion, accumulation of replicative stress, and leukemia [77, 78]. The impaired activity of some E3 ubiquitin ligases has been related to the loss of quiescence and expansion of the HSC compartment.

The RING E3 ligase c-Cbl negatively regulates Notch [79], c-Kit [80], and STAT5 [81, 82], all of which are essential in HSC homeostasis (Fig 2A). c-Cbl-deficient mice have a cell-autonomous increase in HSCs [83]. Similarly, *c-Cbl*-knockout HSCs show enhanced proliferation and reconstitution capacity in competitive bone marrow transplantations. The lack of this E3 ligase promotes self-renewal and aberrant proliferation of stem cells (Fig 2B). All these data support that c-Cbl maintains quiescence of the HSC compartment. Eventually, *c-Cbl*-knockout mice develop myeloid proliferative disorders and acute myeloid leukemia [84]. Interestingly, CBL is mutated in 5–15% of human myeloid proliferative disorders [85–89]. However, more studies are necessary to understand whether those mutations are drivers of leukemia.

Similar to c-Cbl, the HECT E3 ligase Itch is a negative regulator of HSC self-renewal and proliferation [90]. *Itch*-deficient mice show increased numbers of HSC and enhanced self-renewal. These phenotypes are attributed to deficits in the ubiquitylation of Notch (Fig 2A), a well-known target of Itch. Indeed, Notch downregulation in *Itch*-deficient mice can partially rescue the effects on HSCs [29]. However, loss of *Itch* does not promote leukemia.

In contrast, the E3 ligase SCF^{Fbxw7} is mutated in a significant portion of human tumors, including T-cell acute lymphoblastic leukemia (T-ALL) [91–93]. This E3 ligase controls the stability of key hematopoietic regulators such as Notch [94, 95], c-Myc [96, 97], Cyclin E [98, 99], and Mcl-1 [100, 101] (Fig 2A). Germline deletion of SCF^{Fbxw7} results in embryonic lethality due to hematopoietic and vascular defects [95, 101]. Conditional deletion of *Fbxw7* in the hematopoietic system leads to constant HSC proliferation and eventually exhaustion of that population [74, 102] (Fig 2B). As a

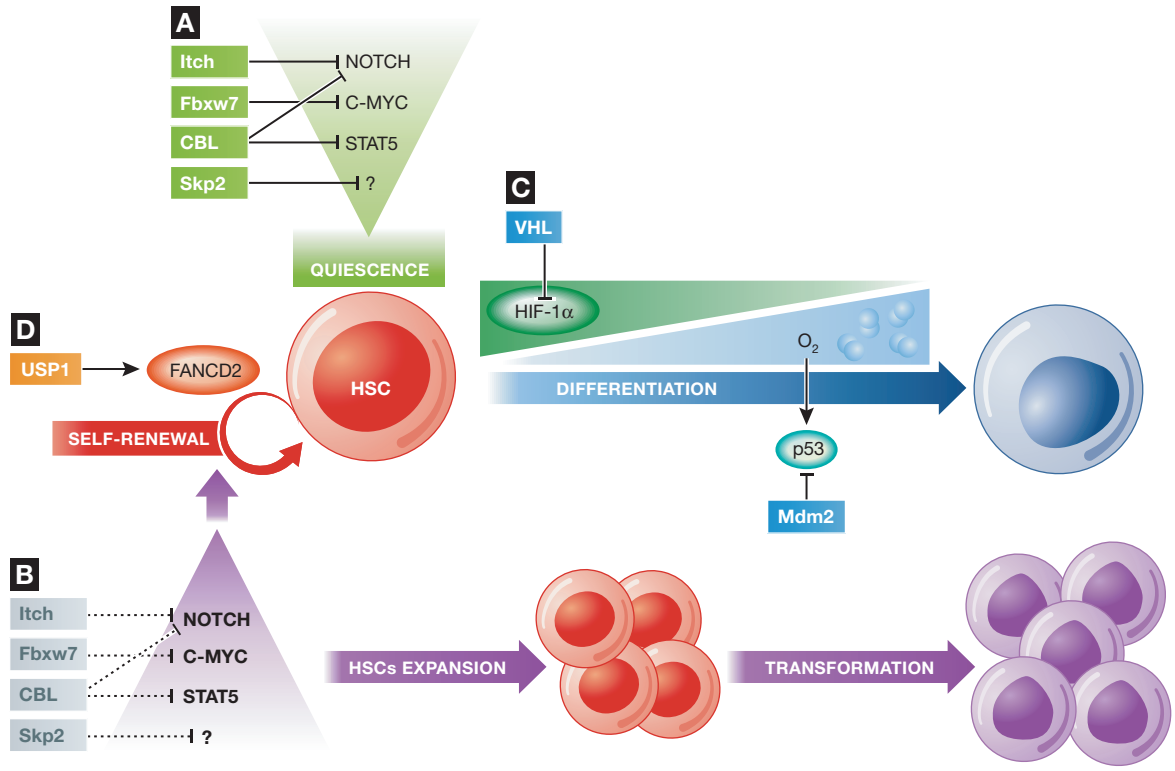

Figure 2. The ubiquitin-proteasome system maintains the balance between quiescence, self-renewal, and differentiation of HSCs.
(A) The E3 ligases Itch, SCF^{Fbxw7},CBL and SCF^{Skp2} maintain the quiescence of the HSC by targeting Notch, c-Myc, and STAT5, among others. (B) Loss of any of those E3 ligases results in self-renewal factor upregulation. As a consequence, HSCs re-enter the cell cycle and divide. The aberrant expansion of HSCs results in hematopoietic exhaustion or leukemia. (C) HSCs need HIF-1α in order to survive in the hypoxic niche. However, during differentiation, they migrate out of the niche. The E3 ligases VHL and MDM2 play an important role in the adaptation to the new environment. HIF-1α must be degraded by VHL for migration and differentiation to occur. Additionally, as increase in ROS activates the p53 pathway, MDM2 controls the levels of p53, promoting cell survival. Impaired activity of USP1, VHL, or MDM2 alters HSC self-renewal and differentiation capacities, resulting in hematopoietic failure. (D) During HSC self-renewal, the DUB USP1 promotes the activation of FANCD2, which is essential for DNA damage repair upon replicative stress.

consequence, a significant percentage of *Fbxw7*-knockout mice develop anemia. Besides the increased proliferation of HSCs, these cells are unable to compete in bone marrow transplantation. Therefore, *Fbxw7* deficiency results in a global loss of quiescence in the HSC compartment and consequently exhaustion of hematopoiesis. Accordingly, genes involved in HSC quiescence are downregulated in *Fbxw7*-deficient LSKs, which are the primitive hematopoietic cells that have the ability to self-renew. This may be due to changes in the stability or transcriptional activity of one or more of the Fbxw7 targets, such as c-Myc. Furthermore, some aged mice develop T-ALL due to the stabilization of c-Myc, which prevents T cells from exiting the cell cycle [74, 103]. Interestingly, Fbxw7 was recently shown to modulate leukemia-initiating cells by the regulation of c-Myc stability [75].

Another important regulator of HSC self-renewal and quiescence is the E3 ligase SCF^{Skp2} (Fig 2A), which triggers ubiquitylation and degradation of cell cycle inhibitors such as p27 [104]. Upregulation of Skp2 has been related to increased proliferation and tumorigenesis [105]. In contrast, depletion of *Skp2* in long-term HSCs (LT-HSCs) can promote the loss of quiescence and proliferation [106, 107] (Fig 2B). While p21 and p27 levels are normal in the *Skp2*-null cells, Cyclin D1 is upregulated. However, Cyclin D1 is not a direct target of Skp2. Therefore, the molecular mechanism driving this proliferative phenotype in stem cells is still unknown.

E3 ligases in HSC differentiation Similar to quiescence, proper control of HSC differentiation is essential for the regulation of hematopoiesis. One of the hallmark characteristics of the HSC niche is its low-oxygen tension, essential for stem cell quiescence and functions [108]. In order to adapt to this hypoxic niche, HSCs have developed appropriate molecular mechanisms for stem cell survival. HIF-1a is the master regulator that orchestrates this hypoxic transcriptional program [109]. When HSCs differentiate, they move out from the HSC niche and HIF-1a is degraded through the E3 ligase VHL [109] (Fig 2C). Indeed, VHL plays an essential role in the exit from quiescence and HSC differentiation. Loss of one or two alleles of *VHL* induces HSC quiescence, determined by both an increase in the total numbers of LSK cells, which contain the HSCs population, and in the numbers of LSKs in G0. As a consequence, there is attenuated differentiation of cells in the peripheral blood. Therefore, VHL is essential for exit from quiescence and the initiation of HSC differentiation.

In addition to the above example, the hypoxic bone marrow niche keeps low levels of reactive oxygen species (ROS). As stem cells move out from the niche to proliferate and differentiate, the concentration of ROS increases. Given that ROS are DNA-damaging agents, cells activate p53 pathways. The RING E3 ligase MDM2 regulates p53 stability, facilitating the survival of HSCs upon those microenvironmental changes. The ablation of MDM2 in the hematopoietic system leads to the stabilization of p53, resulting in cell cycle arrest, senescence, and apoptosis of HSC and progenitor cells [110] (Fig 2C). Several studies have demonstrated that this is a cell-intrinsic effect alleviated by p53 downregulation or treatment with antioxidants [111, 112]. Therefore, MDM2 is required to regulate p53 levels as ROS levels increase when the HSCs migrate away from the niche.

In the adult organism, most HSCs remain quiescent and are thus protected from the DNA damage inherent to DNA replication and from molecular species resulting from cellular metabolism. However, as HSCs re-enter the cell cycle to divide and differentiate, they are exposed to such stress. An important DNA repair mechanism is the Fanconi anemia pathway. Monoubiquitylated FANCD2 is recruited to chromatin to mediate this process [113], and the cysteine protease USP1 deubiquitylates FANCD2 (Fanconi anemia D2) to end this function [114] (Fig 2D). Loss of *Usp1* causes detrimental phenotypes in mice [115, 116], similar to those observed in patients deficient for any of the Fanconi anemia pathway proteins. These include aplastic anemia, developmental abnormalities, and increased cancer susceptibility due to increased genomic instability [117]. Accumulation of DNA damage and apoptosis in the HSC compartment explains bone marrow failure phenotypes in those patients. As a result, the deubiquitylating activity of Usp1 demonstrates its key role in HSC protection against DNA damage. However, it is unclear whether this is only through its regulation of FANCD2 or other targets are also implicated.

Ubiquitylation in epidermal stem cells

The skin is a multilayer organ that protects organisms against external aggressions. It constitutes one of the tissues with the best-characterized hierarchical organization. Stem cells are found in the basal layer of the epidermis and generate several types of progenitors ensuring the high turnover rate of the epithelium. In addition, the bulge region of the hair follicle in mice contains a population of multipotent stem cells that can give rise to all epithelial cell lineages within hair follicles during normal hair growth [118]. Surprisingly, non-hair follicle stem cells can contribute to the formation of hair follicles in response to wounding [119], demonstrating the dynamic characteristics of this system. Although only some of those stem cells remain quiescent, most of the epidermal stem cells can divide symmetrically or asymmetrically in order to self-renew or differentiate. This balance between quiescence, symmetric, or asymmetric cell division is controlled by a number cell-autonomous molecular mechanisms and interactions between stem cells and their microenvironment [120]. As in other tissues, disequilibrium between self-renewal and differentiation results in pathologies like cancer [121].

The canonical Wnt/β-catenin signaling pathway has been implicated in maintaining stem cell homeostasis in epithelial tissues such as the skin, mammary gland, and intestine. Furthermore, deregulation of this pathway often leads to the generation of epithelial cancers [122]. In the absence of active Wnt signaling, intracellular β-catenin is phosphorylated and targeted for proteolytic degradation. High levels of β-catenin lead to hair growth and ectopic hair follicle formation in transgenic mice [123], whereas inhibiting β-catenin blocks the formation of hair during development [124]. In addition, transplant experiments in pre-cancerous murine skin cancer stem cells show that β-catenin signaling is essential for tumorigenesis [118]. Interestingly, the E3 ligase Smurf2 targets Smad7-bound β-catenin for degradation [125] (Fig 3A). Transgenic mice overexpressing *Smad7* show reduced levels of β-catenin and Wnt signaling inhibition, resulting in the delay of hair development. Under physiological conditions, the levels of Smad7 in keratinocytes are low; however, its knockdown leads to overexpression of β-catenin and Wnt signaling. This suggests that low levels of *Smad7* are required in stem cells to maintain a proper balance of β-catenin/Wnt signaling. Accordingly, whether the overexpression of *Smad7*

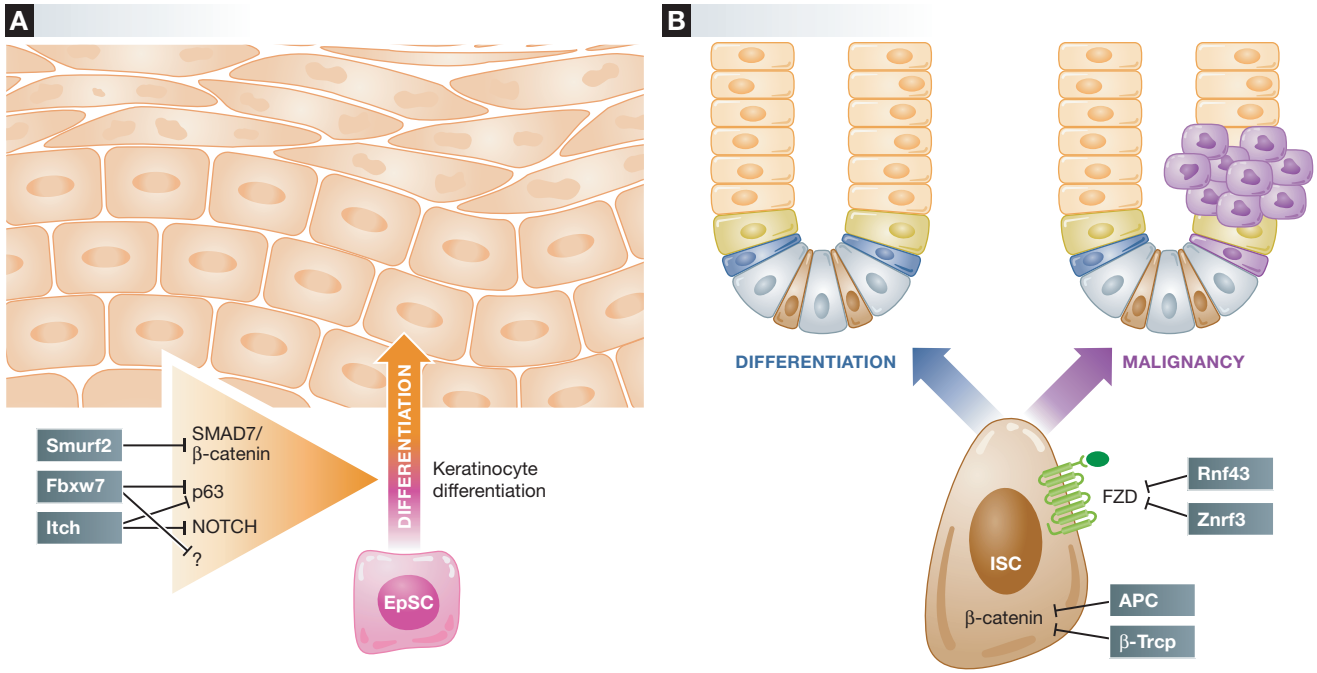

Figure 3. Ubiquitylation maintains the equilibrium between differentiation and transformation in adult stem cells.
(A) The E3 ligases Itch, SCF^{Fbxw7}, and Smurf2 regulate levels of Notch, p63, and β-catenin in epidermal stem cells, mediating keratinocyte differentiation. (B) In intestinal stem cells, the Wnt signaling pathway is modulated by ubiquitylation. Rnf43 and Znrf3 control Frizzled proteins, whereas APC and $SCF^{\beta Trcp}$ regulate β-catenin degradation, collectively controlling differentiation in the intestine. The alteration of this balance due to mutation results in tumor formation.

in β-catenin-dependent cancer stem cells could abolish tumorigenesis is an intriguing possibility that awaits testing.

Another example of ubiquitin-mediated regulation is that of the HECT E3 ligase Itch, which similarly to its function in the hematopoietic system, has important roles in epidermal stem cells. Itch was originally identified through genetic studies aimed to examine the *agouti* locus. The 18H mutation associated with a darker color arises from an inversion disrupting the *agouti* and *Itch* genes [126]. Interestingly, some Itch substrates such us p63, Notch, Gli1, c-Jun, JunB, Erb4 are transcription factors controlling epidermal stem cell maintenance and keratinocyte differentiation [127–130] (Fig 3A). *Itch*-deficient mice develop severe immune deregulation [131] and a thickening of the epidermis [132]. In skin epithelia, the p63 isoform NΔp63α supports the proliferative potential of basal cells. Moreover, its downregulation is required during keratinocyte differentiation [133]. Aberrant accumulation of NΔp63α and Notch during keratinocyte differentiation might partially explain the epidermal hyperproliferative phenotype of *Itch*-null mice. However, the precise role of Itch in epidermal stem cells remains unknown. Furthermore, SCF^{Fbxw7} has been proposed to target p63 during keratinocyte differentiation and DNA damage regulation [134] (Fig 3A). As in other stem cells, SCF^{Fbxw7} could regulate the proliferation of epidermal stem cells.

Ubiquitylation in intestinal stem cells

In the mammalian intestine, rapidly dividing stem cells are confined to the crypts of Lieberkuhn. They continuously self-renew and differentiate to regenerate all tissues every 4–5 days. Alterations in these stem cell functions might result in the expansion of the stem-like cells, adenomas, and cancer [135]. As in skin, Wnt signaling was one of the first mechanisms implicated in the control of gut stem cells by maintaining active cell divisions [136].

Canonical activation of Wnt signaling leads to the stabilization of β-catenin [136]. In the absence of Wnt ligand, β-catenin is sequestered in a multiprotein degradation complex containing the scaffold protein Axin, the tumor suppressor adenomatous polyposis coli APC (Fig 3B) and the kinases CKI and GSK3β. Upon sequential phosphorylation at serine and threonine residues, β-catenin is ubiquitylated by the E3 ligase $SCF^{\beta\text{-}TrCP}$ and subsequently degraded by the proteasome [137] (Fig 3B). Mutations in *APC* or *β-TrCP* result in β-catenin stabilization and nuclear translocation, constitutively activating its transcriptional targets, which induces adenoma formation and colon cancer [138, 139] (Fig 3B). Although mutations in *β-TrCP* have been only found in gastric and endometrial cancer, transgenic mice overexpressing either wild-type or loss-of-function *β-TrCP* develop tumors in a wide variety of organs through β-catenin activation [140, 141]. For these reasons, β-TrCP may be important during stem cell differentiation.

However, β-TrCP is not the only mediator of Wnt signaling. In a recent landmark study, the RING-family transmembrane E3 ligases RNF43 and ZNRF3 were shown to modulate the Wnt pathway, regulating the functions of the LGR5-positive crypt stem cells [142] (Fig 3B). According to this study, RNF43 and ZNRF3 target frizzled receptors for degradation. Deletion of both genes in mouse intestinal epithelium induces rapidly growing adenomas due to an expansion of the LGR5-positive stem cells. Inhibition of Wnt secretion decreased the proliferation of organoids derived from those adenomas [142]. This groundbreaking work provides an excellent

example of signaling cascade modulation by ubiquitylation in stem cell function.

Another E3 ligase that is expressed in the nucleus of the crypt stem cells is SCF^{Fbxw7}. Loss of *Fbxw7* alters intestinal epithelium homeostasis and induces the development of adenomas [143]. In the context of APC deficiency, ablation of *Fbxw7* accelerates intestinal tumorigenesis, promoting an accumulation of β-catenin in adenomas that normally have a long latency. Intestinal alterations and a susceptibility to adenoma formation suggest that lack of Fbxw7 results in the expansion of stem cell crypts. However, further analysis is required to validate the role of Fbxw7 in the intestinal stem cells.

Ubiquitylation in neural stem cells

Multipotent neural stem cells (NSCs) exist both in embryonic and in adult tissues of the nervous system. They reside in various distinct anatomical sites and are able to maintain and specify neurogenesis during early development, as well as throughout post-natal life. Among various signaling pathways that regulate NSC maintenance, differentiation and specification, the Notch pathway is of crucial importance. Notch can inhibit neurogenesis and maintain glial progenitor cells [144, 145]. In this stem cell system, ubiquitin-mediated regulation plays an important role. For instance, the RING E3 ligase Mbi1 shapes Notch regulation by ubiquitylating Notch ligands in neighboring cells. Loss of *Mbi1* results in aberrant Notch activation, which leads to premature neurogenesis [146]. The perturbation of Notch ligand ubiquitylation in the absence of Mbi1 results in their defective endocytosis, sustaining Notch activation [147]. This important function places Mbi1 as an upstream regulator of Notch signaling.

The self-renewal of NSCs must be also regulated by suppression of neural differentiation genes. The transcription factor REST is an important repressor of differentiation toward neurons and constitutes another example of ubiquitin-mediated cell specification. $SCF^{\beta\text{-}Trcp}$ is the E3 ligase that mediates REST proteasomal degradation [148]. Knockdown of *β-Trcp* inhibits neuronal differentiation from ESCs. Similarly, *β-Trcp* expression levels are upregulated during the induction of neuronal differentiation.

Conversely, NSC differentiation must be accompanied by the loss of factors that promote self-renewal. N-myc is an essential transcription factor that promotes cycling and controls the transcriptional program of NSCs. The HECT-type E3 ligase Huwe1 was shown to target N-myc for polyubiquitylation and proteasomal degradation, allowing proper neuronal differentiation [149]. *Huwe1*-knockout ESCs fail to differentiate toward neurons *in vitro* and, similarly, depletion of *Huwe1 in vivo* leads to improper maintenance of stemness characteristics in the mouse brain.

Beyond the described functions in neural development, E3 ligases are often mutated in neurodegenerative diseases. The mouse model of the RING E3 ligase Listerin is an example of the importance of proper ubiquitylation in human disease [150]. Mutant mice for the *lister* gene present defective neuronal and motor functions. Axons and neurons are degenerated, leading to muscle atrophy. Another example of E3 ligases implicated in neurodegenerative disease is the RING ligase Parkin. Mutations in the *Park2* locus, which encodes Parkin, were identified in Parkinson's disease [151]. Parkin was found to bind the E2-conjugating enzyme UbcH8, promoting the ubiquitylation and degradation of the synaptic vesicle-associated protein CDCrel-1 [152]. Several other substrates have been proposed and loss-of-function *Parkin* mutations are associated with a loss of dopamine neurons [153].

Histone ubiquitylation in stem cell function and disease

Although ubiquitylation often leads to the formation of polyubiquitin chains that can ultimately lead to proteasomal degradation, other types of ubiquitin conjugation to substrate proteins do not mediate proteolysis. Examples of such modifications are monoubiquitylation and conjugation of polyubiquitin chains linked through lysines other than K48. These play a pivotal role in stem cell biology, as they can regulate a plethora of different processes, such as regulation of histone function and gene expression, as well as receptor endocytosis and DNA repair [16].

Stem cells impose plasticity in chromatin structure and dynamics, processes that facilitate the rapid establishment of different gene expression patterns after differentiation stimuli. Nucleosomes constitute the basic units of chromatin and are composed of 147-bp DNA fragments surrounding octamers of histones H2A, H2B, H3, and H4, which are present in dimers. The post-translational modification of histone tails can result in alteration of histone physical properties, leading to alterations in protein compaction. In addition, they can serve as a platform for the recruitment of transcription factors, enzymes, and other chromatin-associated proteins [154]. Histone ubiquitylation thus plays substantial roles in the regulation of gene expression programs during stem cell self-renewal or differentiation, mediating transitions in chromatin architecture and organization [155].

As opposed to other modifications such as methylation, phosphorylation, or acetylation that are modest in size, histone monoubiquitylation covalently links a larger approximately 8-kDa protein to histones, affecting chromatin structure. In mammals, H2B-monoubiquitylation (H2B-Ub) on Lys120 is catalyzed by the E3 ligases RNF20 and RNF40 [156], which form a complex, and the E2-conjugating enzymes hRad6A and hRad6B [157] or UbcH6 [158, 159] (Fig 4A). Chromatin immunoprecipitation (ChIP) studies showed that H2B-Ub is closely associated with transcriptionally active regions in chromatin and RNA polymerase II elongation [160–162]. Given that it constitutes a bulky modification associated with open chromatin structure, it was postulated that it causes steric effects to allow the opening of chromatin conformation [163]. H2B-ub has been also shown to regulate nucleosome assembly and disassembly [164]. This is achieved during transcription elongation through the histone chaperone FACT, which can toggle the deposition of H2B-ub. FACT can modulate H2A/H2B removal to facilitate elongation by RNA polymerase II, and it can restore nucleosome assembly, promoting chromatin dynamics and configuration during transcription [159]. The Paf1 transcriptional elongation complex is also involved in H2B-Ub deposition, as it plays an active role in the recruitment of RNF20 to RNA polymerase II among other functions [165].

Given that H2B-Ub modification can have marked effects in gene expression, it plays an important role in stem cell differentiation and adaptation of transcriptional programs. H2B-Ub is significantly upregulated during differentiation of human mesenchymal stem cells (hMSCs) [166] (Fig 4B). Indeed, hMSC differentiation is inhibited upon the depletion of *RNF40*, leading to significant changes in the transcriptional programs of osteoblast and adipocyte lineages.

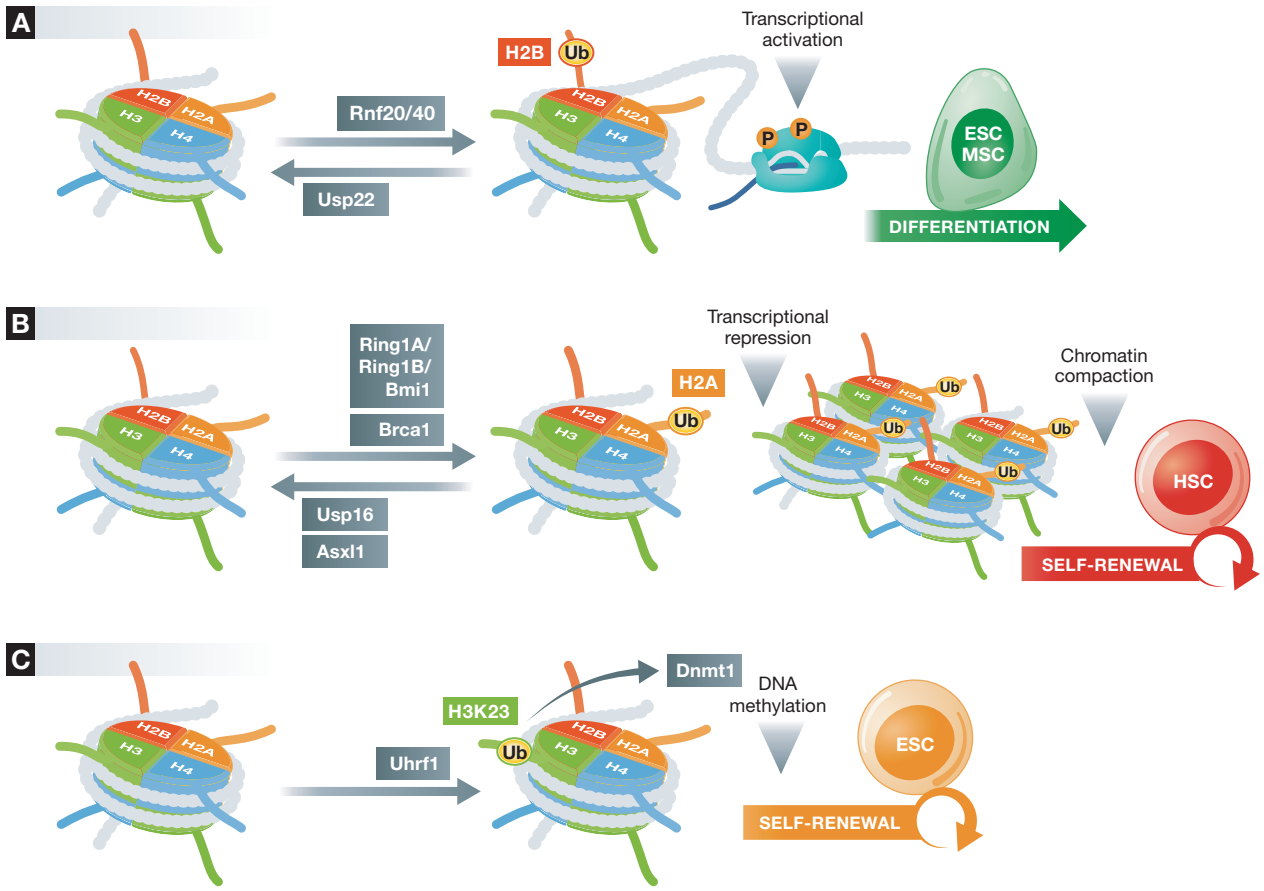

Figure 4. Regulation of chromatin functions in stem cells by ubiquitylation.
(A) H2B ubiquitylation marks open chromatin and facilitates transcription elongation by RNA polymerase II. The E3 ligases RNF20 and RNF 40, and the DUB USP22, control H2B-Ub deposition and transcriptional activation, resulting in ESC and MSC differentiation. (B) H2A ubiquitylation is deposited by either the PRC1 complex, which consists of RING1A/RING1B/BMI1- or the E3 ligase BRCA1, and is associated with transcriptional repression and chromatin compaction. The DUBs USP16 and Asxl1 can reverse this process, regulating the functions of hematopoietic and mammary stem cells. (C) H3K23 ubiquitylation is catalyzed by the E3 ligase Uhrf1. This recruits Dnmt1, which maintains DNA methylation at sites of replication.

Similarly, H2B-Ub is required for the optimal differentiation of embryonic stem cells, and depletion of *RNF20* is associated with impaired induction of differentiation-associated gene subsets [167]. However, the opposite is observed during myoblast differentiation, where deposition of this histone mark is rapidly downregulated, giving rise to myotube formation [168]. Another direct implication of H2B-Ub in gene expression and its implication in cellular identity and morphogenesis is its requirement for the transcriptional activity of Hox genes. Importantly, H2B-Ub was the first histone mark directly implicated in the modification of another histone in a cross-talk mechanism. H2B-Ub is required for H3K4me3 and H3K79me3 formation by complexes containing Set1- and Dot1-histone methyltransferases, respectively [169]. Consistent with that, knockdown of RNF20 leads to a decrease in not only H2B-Ub, but also H3K4 and H3K79 methylation [156].

Besides its positive roles in RNA polymerase II elongation and chromatin compaction, H2B-Ub has also been linked to transcriptional repression. RNF20 is associated with repression of the proto-oncogenes *c-Fos* and *c-Myc* [161]. In addition, in *RNF20*-depleted cells, epidermal growth factor (EGF)-induced genes are de-repressed, suggesting that H2B-ub can have tumor suppressor activity. Similar to E3 ligases, DUBs specific for H2B-Ub can also regulate transcriptional activity and chromatin functions. USP22 is the major H2B-Ub DUB in mammalian cells (Fig 4A). It has been shown to deubiquitylate H2B on promoters, regulating androgen and estrogen receptor-mediated transcription [170]. Furthermore, USP22 is implicated in the activation of c-myc targets in tumor-initiating stem cells [171] and is correlated with tumor metastasis and poor patient prognosis [172, 173]. In *Drosophila*, the H2B-Ub DUB Scrawny (*scny*) is involved in germline, epithelial, and intestinal stem cell maintenance [174]. Aberrant Notch pathway signaling was observed in *scny Drosophila* mutants, suggesting that H2B-Ub functions through the silencing of genes required for differentiation.

Another important mechanism regulating stem cell functions involves H2A ubiquitylation on Lys119. The Ring1B component of the polycomb repressive complex 1 (PRC1) was shown to be the main E3 ligase for this enzymatic function in mammalian cells (Fig 4B), as loss of Ring1b dramatically decreases H2A-ub deposition globally [175–177]. In contrast to H2B-Ub, H2A-Ub plays a repressive role in transcription [175]. In ESCs, it has been associated with poised RNA polymerase II at bivalent genes [178]. Additionally, H2A-Ub correlates with the loss of occupancy of RNA

polymerase II phosphorylated at Ser5 and Ser2, which mark initiation and elongation [158]. During development, H2A-Ub controls Hox gene silencing [176, 179] and X-chromosome inactivation [34, 177], among other functions. Other RING-finger proteins of the PRC1 complex, Ring1A and Bmi1, stimulate H2A-Ub deposition [176, 180], suggesting that Ring1A/Ring1B/Bmi1 act in concert in order to modulate H2A functions. In support of these findings, Bmi1 was shown to be essential for hematopoiesis, as bone marrow HSCs lacking Bmi1 are unable to self-renew properly [69, 70] (Fig 4B). *Bmi1* is also overexpressed or amplified in many leukemias [181]. Similar to HSCs, Bmi1 is known to regulate neural and mammary stem cell self-renewal, preventing differentiation [182–184].

Besides ubiquitylation by the PRC1 complex, other E3 ligases have been proposed to act on H2A to regulate its functions. BRCA1 is a RING-finger E3 ligase with H2A-ub activity [185] (Fig 4B), which is stimulated by its association with BARD1, a RING-finger protein that lacks enzymatic activity [186]. Recent work has associated BRCA1 with satellite regions of the genome [187], suggesting that its loss disrupts constitutive heterochromatin and leads to the deregulation of gene silencing, which suggests a tumor suppressor role. Loss of *BRCA1* is implicated in multiple breast and ovarian cancers [188]. Additionally, *BRCA1* mutations are present in expanded luminal progenitor populations [189], consistent with proposed perturbations in their differentiation program.

In addition to E3 ligases, H2A-specific DUBs are also implicated in the regulation of gene expression during development. USP16 was demonstrated to mediate *Xenopus* embryo patterning through *Hox* gene silencing [190] (Fig 4B). Furthermore, it has been implicated in de-repression of transcription following DNA damage [191]. In addition to USP16, the polycomb protein BAP1 has ubiquitin C-terminal hydrolase activity for H2A-Ub [192]. In *Drosophila*, loss of BAP1 results in diminished deubiquitylation of H2A-Ub and aberrant *Hox* gene de-repression. In addition, its binding partner ASX, also a polycomb protein, is required for H2A-Ub DUB activity. Its mammalian counterpart, *Asxl1*, is often deleted or mutated in a number of hematologic malignancies, and *Asxl1*-knockout stem and progenitor cells present impaired self-renewal [193, 194] (Fig 4B). In addition to monoubiquitylation, H2A and its variant H2AX can be polyubiquitylated with K63-linked chains by the E3 ligases Rnf8 and Rnf168 [195, 196]. This occurs at foci of DNA damage and might serve as a platform in the recruitment of DNA repair-associated proteins.

In addition to H2A and H2B, the other core nucleosome histones, H3, and H4, as well as the linker histone H1, have been proposed to be regulated by ubiquitylation [197, 198]. However, the relevance of most of these modifications in stem cell function remains unclear. Ubiquitylation of H3 at Lys23 (H3K23-Ub) by the RING-finger protein Uhrf1 was recently shown to modulate DNA methylation and DNA replication [199] (Fig 4C). The role of Uhrf1 was known to involve the recruitment of Dnmt1 to sites of hemi-methylated DNA [200, 201] to maintain DNA methylation in mammalian cells [202]. A RING-finger mutant of Uhrf1 expressed in ESCs failed to maintain DNA methylation at DNA replication sites, suggesting a direct implication of H3K23-Ub in proper ESC differentiation.

Collectively, all the above mechanisms of regulation accentuate the importance of proteolytic-independent functions of ubiquitylation in stem and progenitor cell functions.

Sidebar A: Regulation of symmetric and asymmetric stem cell divisions by ubiquitylation

The *Drosophila* and *C. elegans* embryos constitute a powerful tool to study the mechanisms of asymmetric cell division during early development. Several ubiquitin-mediated pathways have been recently implicated in these processes. The E3 ligase Neuralized (Neur) has been shown to regulate epithelial cell polarity [211]. Neur ubiquitylates the Notch ligand Delta, promoting its internalization. In addition, *bearded* can inhibit Neur, restricting its activity to the mesoderm and contributing to the establishment of cell polarity. In an analogous function, NEUR also promotes NOTCH DL internalization in the apical zone of the polarized human kidney cell line MDCK [212]. However, the specific roles of Neur during mammalian development *in vivo* and whether this E3 ligase is important in the adult epithelial cells have not been explored yet.

The asymmetric inheritance of cellular components in *C. elegans* is controlled by the interplay between PIE-1 and MEX-5. PIE-1 represses transcription by promoting the expression of germline-associated genes [213]. MEX-5 on the other hand, through activation by ZIF-1 and phosphorylation by PAR-1 [214], forms an E3 ligase complex that degrades PIE-1, establishing segregation and anterior–posterior cytoplasm specification [6].

In addition, the E3 ligase SCF^{Slimb} ($SCF^{\beta Trcp}$ in mammals) was shown recently to regulate asymmetric division in *Drosophila* neuroblasts [215]. Slimb is able to associate with kinases Sak and Akt, promoting their ubiquitylation and inhibiting ectopic neuroblast formation. Supporting this notion, *β-Trcp* is often deleted in human gliomas with a simultaneous activation of Akt signaling [216]. SCF^{Slimb} was also implicated in the degradation of Oskar in the *Drosophila* oocyte [217]. In the latter case, Par-1 was shown to be the priming kinase, which allows Gsk3 to phosphorylate an Oskar degron in order to allow degradation by SCF^{Slimb} and establish polarity.

These examples demonstrate the importance of ubiquitin-regulating mechanisms in the balance between symmetric or asymmetric stem cell divisions that establish early tissue specification.

Ubiquitin regulation in cancer stem cells: potential therapeutic targets

Cancer stem cells (CSCs) are defined as a population that have the capacity to self-renew, differentiate, and regenerate the cells that originated the tumor. The concept of CSCs remains controversial and probably the role that CSCs play in tumor biology depends on individual types of malignancy. However, emerging evidence suggests that cancers consist of heterogeneous populations in which cancer-initiating cells can regenerate the bulk of the tumor [203]. Therefore, the concept of CSCs will undoubtedly help us understand tumor biology and design novel therapeutic strategies.

As explained for different stem cell systems above, ubiquitylation controls self-renewal or differentiation through the regulation of different substrates. Acquisition of self-renewal capacity is one of the first tumorigenic events in most tumors. In fact, alterations of the ubiquitin pathway are known to promote tumorigenesis in mouse models by inducing stem cell-like phenotypes [29, 204, 205]. One of the most studied examples is the E3 ligase SCF^{Fbxw7}. As discussed above, Fbxw7 can target several oncoproteins, such us Notch, c-Myc, and Cyclin E. Thus, it is not surprising that Fbxw7 acts as a tumor suppressor. In fact, *Fbxw7* is located on chromosomal region 4q32, which is frequently lost in tumors. Moreover, Fbxw7 is mutated in several malignancies, such as cholangiocarcinoma, T-ALL, colon, endometrium, and stomach cancers [91, 99, 206, 207].

Sidebar B: Quantitative ubiquitin proteomics to study dynamic changes of stem cell identity

In order to delve into the systemic details of post-translational regulation of cellular plasticity, specific substrate–enzyme interactions have been interrogated in the context of stem cell differentiation or cellular transformation. A lot of these studies have been performed primarily using epitope tagging and affinity-based approaches and have been very informative about the nature and specific details of the enzymatic functions orchestrating these processes [218]. However, advances in the field of mass spectrometry in recent years enabled the use of proteome-wide studies in order to understand the dynamics of signaling networks, providing a holistic overview of cellular identity changes [219].

These powerful proteomic tools have been utilized in the past to study phosphorylation, acetylation or other small protein modifications in a system-wide manner [220]. However, ubiquitylation poses additional challenges in comparison, considering its bulk size and chain branching. Nevertheless, a novel tool that has revolutionized this field is the use of monoclonal antibodies that recognize di-glycine moieties linked by an isopeptide bond to lysine side chains of proteins [221]. These epitopes, which constitute the remnants of ubiquitylation events after tryptic digestion, can be biochemically isolated and subjected to mass spectrometry in order to identify whole-proteome ubiquitin signatures, while providing site-specific information [222].

Importantly, this methodology can be effectively combined with SILAC strategies, which allow for metabolic labeling of proteins in culture using isotopic amino acid variants [223]. This powerful approach and methodology allows the detailed study of changes in ubiquitin conjugation in a quantitative manner in response to different stimuli. Such technology has been particularly useful to delineate the temporal changes of ubiquitylation in stem cell differentiation and reprogramming, allowing the correlation of relative protein abundance of self-renewal factors with differentiation timing [44]. Additionally, it provides valuable information with regard to dynamics in site specificity and protein turnover after differentiation stimuli. Additional examples of this application include the identification of substrates for Cullin-RING ligases (CRLs) [224]. Taken together, the applications described above can expand our knowledge on missing E3 ligase-substrate pairs and also on protein network dynamics during stem cell maintenance or differentiation.

Ablation of *Fbxw7* in the hematopoietic compartment results in T-ALL, whereas deletion in the intestine promotes the development of adenomas. The upregulation of the Notch pathway in both systems explains the acquisition of self-renewal capacity in the transformed cells. Moreover, in the hematopoietic compartment, stabilization of c-Myc in *Fbxw7* mutant mouse models has been shown to promote cancer-initiating cell populations [75] Interestingly, treatment of those mice with c-Myc inhibitors leads to T-ALL remission.

Furthermore, the important roles of ubiquitylation in stem cell and cancer stem functions illustrate the importance of its machinery as therapeutic targets. The best example in cancer therapy is Bortezomib, or Velcade, which inhibits protein degradation by the proteasome and is approved in the United States for the treatment of relapsed multiple myeloma and mantle cell lymphoma. However, as Bortezomib affects all ubiquitin-tagged proteins, therefore lacking substrate specificity, it may cause undesired effects. In contrast, inhibition of one E3 ligase would lead to the accumulation of only a small number of substrates. Efforts to identify drugable cancer-specific targets include screening for altered requirements of ubiquitylation between stem and cancer stem cells. For instance, the comparison of primary glioblastoma cancer stem cells with neural stem cells revealed 28 E3 ligases whose downregulation promotes CSC differentiation or apoptosis [208]. A model enzyme for drug targeting is MDM2, which controls p53 degradation, and it is associated with several malignancies. Cis-imidazolines and spirooxindoles can disrupt the interaction between p53 and MDM2, and these compounds are currently in clinical trials to explore their activity against human tumors [209]. Additional efforts include *in silico* screens to identify compounds that inhibit SKP2 [210]. Inhibiting SCF^{SKP2} E3 ligase activity impairs the proliferation of a wide range of cancer cell lines. Additionally, it diminishes the prostate cancer stem cell properties of PC3 cells. Collectively, the above studies stress the importance of ubiquitylation in tumor-initiating cell biology. Manipulation of ubiquitin-regulating mechanisms can alter the oncogenic properties of cancer stem cells, leading to effective therapies.

Sidebar C: In need of answers

(i) As only few enzyme–substrate pairs have been characterized in stem cell functions, which additional E3 ligases and DUBs control differentiation or self-renewal in various stem cell systems?
(ii) How can technical advances in the field of proteomics help us understand ubiquitylation events in small stem cell populations *in vivo*?
(iii) How can we develop small-molecule inhibitors for specific components of the ubiquitin pathway? Given that enzymes often regulate multiple substrates in different cellular contexts, how can these strategies change the properties of specific stem cell systems?
(iv) Stem cells often represent < 1% of the total cell population in adult tissues. Can we identify ubiquitylation events that regulate the expansion of stem cell populations?
(v) Can we identify "ubiquitin codes" in stem cells? Can different ubiquitin linkages and branching decipher alternative outcomes in the regulation of quiescence or differentiation?
(vi) Which enzymes differentially control tumor initiation, progression and relapse? As the field needs additional *in vivo* proof that ubiquitin pathways are important for these functions, what is the distinction between substrates in malignancies?
(vii) Which additional non-proteolytic functions of ubiquitylation result in alterations in membrane receptor repertoire, localization of factors and chromatin landscape during stem cell differentiation? How are these processes deregulated in cancer stem cells?

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Conclusions and future directions

Since its identification, the UPS has emerged as an important regulator of different processes through the control of protein stability. Additionally, the identification of proteolytic-independent functions has linked the ubiquitin system to the regulation of signaling networks and epigenetic mechanisms. Together, the proteasome-dependent and independent functions of the ubiquitin system play an important role in stem cell quiescence, self-renewal, and differentiation. Only a few of the numerous E3 ligases and DUBs encoded in the human genome are well characterized. In addition, little is known about how others regulate stem cell fates. Therefore, the identification of the role and targets of additional enzymes could help us understand additional characteristics of stem cell biology. Moreover, identifying the mechanisms of deregulation of specific enzymes that impinge on tumor initiation and progression could lead to effective therapies against additional types of cancer. As this fast-moving field stands at the crossroads of proteomics, stem cell biology, and therapeutics, there are increasing expectations for effective manipulation of cellular systems and the discovery of new concepts in protein modification in cell plasticity.

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Conflict of interest
The authors declare that they have no conflict of interest.

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