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Review

Cross-talk between redox regulation and the ubiquitin–proteasome system in mammalian cell differentiation ☆

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ABSTRACT

Background: Embryogenesis and stem cell differentiation are complex and orchestrated signaling processes. Reactive oxygen species (ROS) act as essential signal transducers in cellular differentiation, as has been shown through recent discoveries. On the other hand, the ubiquitin–proteasome system (UPS) has long been known to play an important role in all cellular regulated processes, including differentiation.
Scope of review: In the present review, we focus on findings that highlight the interplay between redox signaling and the UPS regarding cell differentiation. Through systems biology analyses, we highlight major routes during cardiomyocyte differentiation based on redox signaling and UPS modulation.
Major conclusion: Oxygen availability and redox signaling are fundamental regulators of cell fate upon differentiation. The UPS plays an important role in the maintenance of pluripotency and the triggering of differentiation.
General significance: Cellular differentiation has been a matter of intense investigation mainly because of its potential therapeutic applications. Understanding regulatory mechanisms underlying cell differentiation is an important issue. Correspondingly, the role of UPS and regulation of redox processes have been emerged as essential factors to control the fate of cells upon differentiation. This article is part of a Special Issue entitled Redox regulation of differentiation and de-differentiation.

1. Introduction

Cellular differentiation is induced by gene expression changes triggered by finely tuned metabolic signals. Although growth factor-specific metabolic cascades that modulate gene expression are the classical signals for differentiation, the effect of O_2 availability on stem cell function or during differentiation is a matter of intensive investigation, as depicted in the current literature [1–5]. Low oxygen levels during cell differentiation were first described during mammalian embryogenesis [6]. More recently, studies with stem cells have firmly established that these cells reside into low oxygen[1] niches and that their self-renewal ability is supported in this condition. However, stem cell differentiation also takes place in vitro at very high O_2 concentrations (21%). In fact, varying O_2 levels induces diverse phenotypes during the differentiation of stem cells [7]. In conclusion, changes in O_2 concentration do not trigger cell differentiation, though it is an important factor in determining the fate of cells upon differentiation. The hypoxic condition is regulated in cells and tissues by the hypoxia-inducible factor (HIF) [8]. Decreasing oxygen concentrations results in an exponential increase in HIF expression. In turn, HIF transactivates several genes whose products are involved in the metabolic adaptation to low oxygen availability (e.g., glucose transporters and glycolytic enzymes) and vascular (re) modeling to increase oxygen delivery (e.g., erythropoietin and the vascular endothelial growth factor). Other transcriptional factors that sense the O_2 concentration and promote metabolic adaptation are nuclear factor-κB (NF-κB) and activator protein-1 (AP1). Some of the mechanisms by which low O_2 influences stem cell behavior rely on

Abbreviations: AGE, advanced glycation end products; AP1, Activator protein-1; BER, Base excision repair; CM, Cardiomyocytes; DEG, Differential expressed gene; ERAD, Endoplasmic Reticulum-Associated Degradation; ESC, embryonic stem cells; GSH, reduced glutathione; GSSG, oxidized glutathione; HIF, hypoxia-inducible factor; HSC, hematopoietic stem cells; MSC, mesenchymal stem cell; NER, nucleotide excision repair; NF-κB, nuclear factor-κB; PRDX, peroxiredoxin; ROS, reactive oxygen species; SCF^{Fbxw7}, SKP1-cullin-F-box Fbxw7; SOD1, superoxide dismutase; TFAM, mitochondrial transcription factor A; TSC, tuberous sclerosis complex; UBL, ubiquitin-like proteins; UPR, unfolded protein response; UPS, ubiquitin–proteasome system

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[1] As defined elsewhere [2], normal O_2 concentrations (physiological normoxia) for mammalian cells are those in the range of 2–9%, and low O_2 or physiological hypoxia is defined as concentrations below that range. The O_2 atmospheric concentration is 21%.

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activation of the Notch signaling pathway by HIF-1α to maintain an undifferentiated state, whereas expression of OCT-4, which controls stem cell renewal and pluripotency, is induced by HIF-2α. As discussed below, upon differentiation, a shift in O_2 utilization occurs as mitogenesis increases, resulting in ROS production. In such conditions, HIF is suppressed by increased degradation and inhibited expression.

Regarding redox metabolism during differentiation, the challenge is to understand how cells adapt to O_2 shifts during and after differentiation and which redox mechanisms underlie this process. Data accumulated thus far show that ROS function as signaling molecules to promote stem cell differentiation into cells of multiple lineages. The mechanisms underlying such process are regulated by intrinsic redox control in stem and progenitor cells through various redox signaling pathways. Mechanisms of redox signaling are based on specific redox reactions, mainly through oxidation and reduction of protein cysteine residues. Regardless, it is well known that ROS cause oxidative damage, and the redox modification of proteins can modulate their structure and activity, leading to the modulation of essential pathways in cell metabolism. Cell effectors susceptible to redox modulation are mainly kinases, phosphatases and transcription factors.

As previously noted [9], the increase in ROS levels as a population of stem cells differentiates can be taken as a paradigm. Low levels of ROS are required for quiescence and stem cell maintenance, whereas ROS induction would lead to proliferation and differentiation programs. Correspondingly, reports in the literature suggest an important intracellular pro-oxidative environment during differentiation because of increased mitochondrial ROS production. Increased production of either mitochondrial superoxide or cellular levels of other reactive oxygen species, together with the downregulation of major antioxidant genes, has been reported in human embryonic stem cell (ESC) lines upon differentiation [10]. Mitochondrial ROS generation was also shown to perform an important role in keratinocyte differentiation [11]. Mitochondrial transcription-factor A (TFAM)-deficient mice presented impaired epidermal differentiation and hair follicle growth. TFAM is required for the transcription of mitochondrial genes encoding electron transport chain subunits. To prove that mitochondrial ROS production underlies this process, keratinocytes were treated in vitro with antioxidants that inhibited differentiation. However, differentiation capability was recovered upon the application of exogenous H_2O_2. In hematopoietic stem cells (HSC), the deletion of the tuberous sclerosis complex (TSC), a mammalian target of the rapamycin mTOR pathway, drives quiescent mouse HSCs to differentiate and undergo increased mitogenesis, consequently resulting in elevated levels of ROS [12]. TSC deletion was shown to dramatically reduce hematopoiesis and HSC self-renewal, indicating that by repressing ROS production, HSCs can be maintained in a quiescent state. Regarding other redox regulators, FOXO-mediated transcription of genes encoding antioxidants has been implicated in the maintenance of stem cell populations, as deletion of Fox1, 3 and 4 in HSCs resulted in differentiation [13]. According to metabolomic studies [14], the activation of the oxidative metabolism during ESC differentiation was detected by changes in the GSH/GSSG ratios and ascorbate levels during the process of differentiation. Hydrogen peroxide levels were also shown to play a decisive role in either the maintenance or differentiation of HSCs [15]. At low levels, H_2O_2 maintains the quiescence of those cells, whereas high levels trigger proliferation, senescence or apoptosis. Human mesenchymal stem cell (MSC) differentiation to adipocytes or osteoblasts is regulated by specific redox states, including increased GSSG potential, as cells go through differentiation [16]. Increased ROS levels were also described upon the differentiation of neural stem cells [17,18]. These findings exemplify the increase in ROS production upon cellular differentiation.

Maintenance of the pluripotent state as well as differentiation are finely regulated processes at different levels. As proteins are considered to be major effectors of such processes, investigation of protein turnover (expression, translation and degradation) and post-translational and oxidative modifications are important matters for understanding differentiation. Baharvand and co-workers [19] found that 54 protein spots quantitatively changed and another 14 spots were qualitatively modified among human ESC cell lines during differentiation. In the same study, a large number of proteins found at high concentration were identified as chaperones, heat shock proteins, proteins of the ubiquitin/proteasome system, and oxidative stress responsive proteins, suggesting a pro-oxidative environment and the ability of these cells to resist oxidative stress upon differentiation.

Intracellular protein degradation is a regulatory process of protein function, as it determines protein half-life and, importantly, prevents the accumulation of damaged proteins and/or of their aggregation. In this scenario, the proteasome is a major player, as it finely controls protein half-life, mainly through the degradation of poly-ubiquitylated substrates and unfolded or oxidatively modified proteins.

The present review focuses on the proteasomal role in the redox metabolism during cell differentiation.

2. The ubiquitin–proteasome system

The ubiquitin–proteasome system (UPS) is highly conserved in eukaryotes, from yeast to humans. Protein ubiquitylation is a finely tuned regulatory process of controlling intracellular protein half-life through degradation. The UPS is responsible for degradation of those proteins involved in processes related to the regulation of cell cycle, antigen presentation through MHC-I complexes, and signaling cascades that maintain homeostasis or trigger death. Proteasomal protein degradation plays two important roles: control of regulatory proteins and clearance of misfolded and oxidatively damaged proteins. Clearance of misfolded proteins, namely Endoplasmic Reticulum-Associated Degradation (ERAD), is accomplished through poly-ubiquitylation [20]. On the other hand, degradation of oxidatively modified proteins and of proteins presenting unstructured domains is attributed to the proteasome through a ubiquitin-independent process [21,22].

2.1. Protein ubiquitylation

The process of ubiquitylation involves three steps of events, as follows: ATP-dependent ubiquitin activation by E1 enzymes followed by ubiquitin conjugation to E2 (conjugating) enzymes and, finally, the transfer of ubiquitin to protein substrates through E3 (ligases) proteins. The last step can be either enzymatic by transferring the ubiquitin moiety to E3-bound substrates (HECT E3 enzymes) or occur through the direct transfer of ubiquitin to the protein substrate by scaffolding of substrates to RING or U-BOX E3 proteins [23]. Ubiquitin (8.5 kDa) is one of the most conserved eukaryotic proteins and is encoded in human DNA by the poly-ubiquitin genes Ubb and Ubc and the constitutive monomeric ubiquitin ribosomal fusion gene [24]. As ubiquitin is highly conserved, only two human E1 isoforms transfer ubiquitin to dozens of E2 isoforms. On the other hand, E3 proteins are abundant, as they specifically interact with protein substrates. Approximately 1000 are predicted in the human genome [24].

The protein substrate is bound to the ubiquitin molecule through the interaction between the ε-NH_2-Lys of the substrate and the C-terminus Gly residue of ubiquitin. The poly-ubiquitin chain is built up through the successive addition of ubiquitin molecules via the C-terminal Gly residue of one ubiquitin to a Lys residue of another ubiquitin molecule. Poly-ubiquitylation can generate multiple topological chains depending on which, among seven, ubiquitin-Lys residues (K6, K11, K27, K29, K33, K48, and K63) participates in the poly-ubiquitin chain formation. The orchestrated interactions between E3-substrates and E2-E3 complexes direct the ubiquitin-Lys residue, one of the seven cited above, to form the poly-ubiquitin chain that then determines the topological arrangement of the poly-ubiquitin chains [23,25]. Poly-ubiquitin chains through K48 linkages are related to the degradation of substrate, whereas other topological chains regulate substrate fate in diverse ways, such as protein degradation, apoptosis, signal transduction, gene transcription, DNA

repair, cell cycle progression, immune responses, virus budding, protein trafficking, and receptor and channel endocytosis. Other small proteins, highly similar to ubiquitin, called ubiquitin-like proteins (UBL) can also modify protein substrates [26]. The fate of substrates modified by UBLs is generally related to other regulatory processes.

2.2. The proteasome

The proteasome is a multimeric and multicatalytic protease composed of a central cylindrical-shaped unit called 20S, which is flanked on one or both sides by regulatory units. The 19S regulatory unit is the most abundant and is responsible for recognizing poly-ubiquitylated substrates [27]. The 19S unit (900 kDa) is composed of 19 subunits with a topological distribution that resembles a base and a lid. An ATPase hexameric ring is part of the base together with four other subunits, among them two ubiquitin receptors. The lid consists of seven scaffold subunits (Rpn3, 5, 6, 7, 9, 12 and 15), the deubiquitylating enzyme Rpn11, and the Rpn8 subunit. Near atomic resolution (subnanometer-resolution) of the topological structure of the 19S regulatory unit has been matter of intensive investigation [28–30]. Such studies have answered important questions regarding the location and function of many subunits and the dynamics of the 19S interaction with the 20S core particle and substrates. However, the location and role of many 19S subunits are still ambiguous.

The 20S proteasome is composed of two identical and central heptameric rings called β, which is flanked on both sides by two identical heptameric rings termed α, which are involved in the control of the 20S gating. Three catalytic sites are located in the central β units (β1, β2 and β5), totalizing six sites. The 20S is a threonine protease, and the catalytic sites cleave the protein substrate after acidic (β1), basic (β2) and hydrophobic (β5) residues. These activities are called chymotrypsin-like, trypsin-like and caspase-like, respectively. Protein fragments generated comprise 3 to 25 amino acids.

Alternative proteasomes are described such as the immune and thymus proteasomes, both of which contain alternative catalytic subunits. The immune proteasome contains β1i, β2i and β5i, while the thymus proteasome contains the thymus-specific subunit β5t [31]. In both examples, respective constitutive catalytic subunits are replaced by these specific subunits, thereby changing the overall activity of the proteasome. As recently shown, when either the immune or thymus subunits are co-expressed in cells together with the standard subunits, they are preferentially selected for the assembly of the core particle over the standard counterparts [32]. The term *immune* proteasome was coined because the induction of the β1i, β2i and β5i subunits was first verified upon γ-IFN treatment [33]. Therefore, the immune proteasome is found in several conditions independent on the immune response, as discussed below. More recently, an alternative 20S form expressed in male germ cells that replaces the constitutive α4-subunit by an alternative subunit (α4s) has been described [34]. The functional role of this replacement has yet to be described.

The UPS participates in the fine regulation of cellular events because protein poly-ubiquitylation is a highly regulated process in all steps, including modulation in an oxidative fashion, as all steps of poly-ubiquitylation are carried out by proteins that are dependent on Cys residues (E1, E2 and–E3). Protein poly-ubiquitylation is triggered by post-translational modifications, e.g., phosphorylation [35]. Additionally, poly-ubiquitylation is dictated by the N-end rule, in which the identity of destabilizing N-terminal residue(s) next to the N-terminal Met, termed degrons, determines an enzymatically orchestrated mechanism driving ubiquitylation [36].

2.3. Proteasomal ubiquitin-independent degradation

Studies using proteomic techniques indicate that 20% of intracellular proteins undergo proteasomal degradation independent of poly-ubiquitylation [37,38]. According to the N-end rule [36,39], proteins can be modified by mild oxidation, which may augment their ubiquitylation due to oxidation-induced conformational changes to expose specific residues as targets for ubiquitylation. However, the main hypothesis to date is that the majority of oxidized proteins undergo degradation through the 20S proteasome independent of poly-ubiquitylation [40]. The first lines of evidence were presented in the 1980s with the observation that the incubation of oxidized proteins with erythrocyte extracts resulted in degradation independent of ATP and, most importantly, that the non-oxidized form of model proteins was not degraded in the same conditions [41]. Since then, degradation of oxidized proteins by the 20S proteasome has been extensively investigated through in vitro approaches and in cellular models [21]. Proteins go through oxidation by several mechanisms, generating amino acid side chain modifications [42] and consequent mild loss of secondary structure and exposing hydrophobic patches that can interact with the 20S proteasome [40]. To date, very few mechanisms of protein repair have been described. These are centered on repair of side chains of sulfur-containing amino acids (Cys and Met; [43]. Degradation is therefore claimed to be the cellular mechanism that deals with oxidized proteins. This is an interesting mechanism of defense, as it overcomes the time and energy consumption kinetic of protein poly-ubiquitylation and further degradation, as previously demonstrated [44]. Many indirect data in the literature show the 20S proteasome as the main player in the degradation of oxidized proteins [21], e.g., a large pool of the free 20S proteasome in several cell lines (around two third; [27]), uncoupling between 19S and 20S particles during oxidative stress [45–48], transient inactivation of enzymes involved in protein ubiquitylation and deubiquitylation [49–51], higher susceptibility of the 19S regulatory particle to oxidation compared to the 20S core [45,52], and no preference for the ubiquitylation of oxidized proteins [53].

Although the presence of a free pool of the 20S proteasome is predicted inside cells [27], no systematic study has been reported to evaluate this pool upon differential redox status of cells. An important issue centers on the gating conformation of such pools. The latent 20S proteasome form is believed to be in a closed conformation, which does not support the entrance of folded proteins [54]. However, as extensively investigated, a higher surface hydrophobicity of oxidized proteins is determinant for interaction with the 20S proteasome [38,55–58]. More recently, modulation of the yeast 20S proteasome gating through an oxidative post-translational modification, namely S-glutathionylation, was demonstrated [43,59]. These studies showed gate opening of the 20S proteasome when the 76 Cys residue in the α5-subunit is S-glutathionylated and closure when this Cys residue is in the reduced form. The open conformation was shown to degrade oxidized or unstructured protein models at higher rates. Most likely, this mechanism is a regulatory modification to cope with decreased cellular reductive abilities when the oxidized pool of proteins is increased [43].

2.4. The 11S regulatory unit and immune 20S proteasome as players in the degradation of oxidized proteins

In recent years, participation of immune proteasomal forms, the 11S regulatory unit and the 20S-modified proteasome carrying the catalytic immune subunits (β1i, β2i and β5i), has been reported in cellular defense against oxidative stress [60–66] and in the induction of the immune 20S proteasome in tumor cell lines, as illustrated [67].

The 11S unit, also called PA28αβγ or REG αβγ [68], is expressed in higher eukaryotes. PA28α and PA28β preferentially form a heteroheptamer, while PA28γ is a homoheptamer. Many studies have implicated PA28αβ in the production of peptides for MHC-class I presentation, although the mechanistic basis for this function remains elusive [69]. The 11S activator (PA28αβ heptamers) stimulates the degradation of peptides, while the γ-homoheptamer has been implicated in the degradation of regulatory folded proteins [70]. The 11S unit was previously considered to be an immune regulatory particle because it was first described upon γ-IFN challenge [71]. However, PA28αβ and

the 20S immune proteasome are induced during transient adaptation to oxidative stress [63,65,66]. The redox-responsive transcription factor Nrf2 is a key element for the induction of standard 20S subunits and of the PA28αβ regulator during adaptation to oxidative stress. The immune 20S proteasomal subunits are also induced during the oxidative stress response, though the regulation of their increased expression is not known [65]. Correspondly, during IFN-induced oxidative stress the immune 20S proteasome is upregulated [72]. Most likely, the expression of these immune isoforms is to increase the rate of protein degradation to maintain proteostasis during IFN challenge [73,74].

3. Proteasomal protein degradation during cell differentiation

Protein degradation is an important metabolic regulator of protein half-life, which, in turn, is important for regulation of almost every cellular function. As mentioned above, the main proteolytic system inside cells is the ubiquitin–proteasome. Undoubtedly, protein poly-ubiquitylation is the most important regulatory mechanism to finely control protein function. Accordingly, important findings on the control of differentiation by protein poly-ubiquitylation have been reported, as discussed below. Fig. 1 briefly summarizes the role of UPS during differentiation.

3.1. The regulation of cell differentiation by poly-ubiquitylation

Protein ubiquitylation modulates the stability and function of an important set of regulatory factors involved in biological processes such as entrance into and progression of the cell cycle [75]. Alterations in the abundance of some transcription factors result in the re-direction of cellular fate, e.g., from a pluripotent state to differentiation. At the level of post-translational protein modification, ubiquitylation specifies processes, such as protein poly-ubiquitylation, to control the pool of signaling proteins and transcription factors in stem cells. Some of these transcription factors are known as pluripotency factors, associated with the maintenance of pluripotency and the balance between stem cell self-renewal and differentiation. The abundance of pluripotency factors (Oct-4, Nanog and c-Myc) is regulated by poly-ubiquitylation to specify differentiation processes [76].

E3 ligases have their function and protein targets regulated by tissue-specific factors in a developmentally regulated step-wise fashion. Illustrating that process upon differentiation, the E3 ligase, namely SCF^{Fbxw7} (SKP1-cullin-F-box Fbxw7), utilizes different substrates depending on cell type and the subsequent process. Silencing SCF^{Fbxw7} expression in ESC was shown to inhibit differentiation and to increase cell reprogramming by stabilizing the pluripotency factor c-Myc [77–79]. Silencing SCF^{Fbxw7} in the mouse brain inhibited neural stem cell differentiation and increased progenitor cell death due to c-Myc and Notch up-regulation [80,81]. Moreover, SCF^{Fbxw7} inactivation impaired the differentiation of stem cells from hematopoietic, intestinal and liver lineages [77,82–84]. These examples illustrate that SCF^{Fbxw7} is a key component of differentiation and self-renewal of various stem cells lineage, which, in turn, might be attributed to differential regulation of this E3 ligase and/or of its substrates according to tissue specificity.

Osteogenesis is well-known model of cellular differentiation regulated by the UPS [85]. In addition to the regulation of the pluripotency factors mentioned above, the abundance of signaling proteins and transcriptional factors in the osteogenesis pathway is finely regulated. The E3 ligases Smurf1 and c-Cbl negatively regulate osteoblast differentiation by increasing the degradation of signaling proteins as JunB, Runx2 and MEKK by Smurf1 and STAT5 by c-Cbl [86–91]. Moreover, other E3 ligases play important role in osteogenesis by regulating Runx2, Osx, SMADs, and OASIS, among other proteins (Table 1).

Table 1 summarizes the major findings regarding the role of UPS through E3 ligases upon differentiation in several cellular lines and tissues.

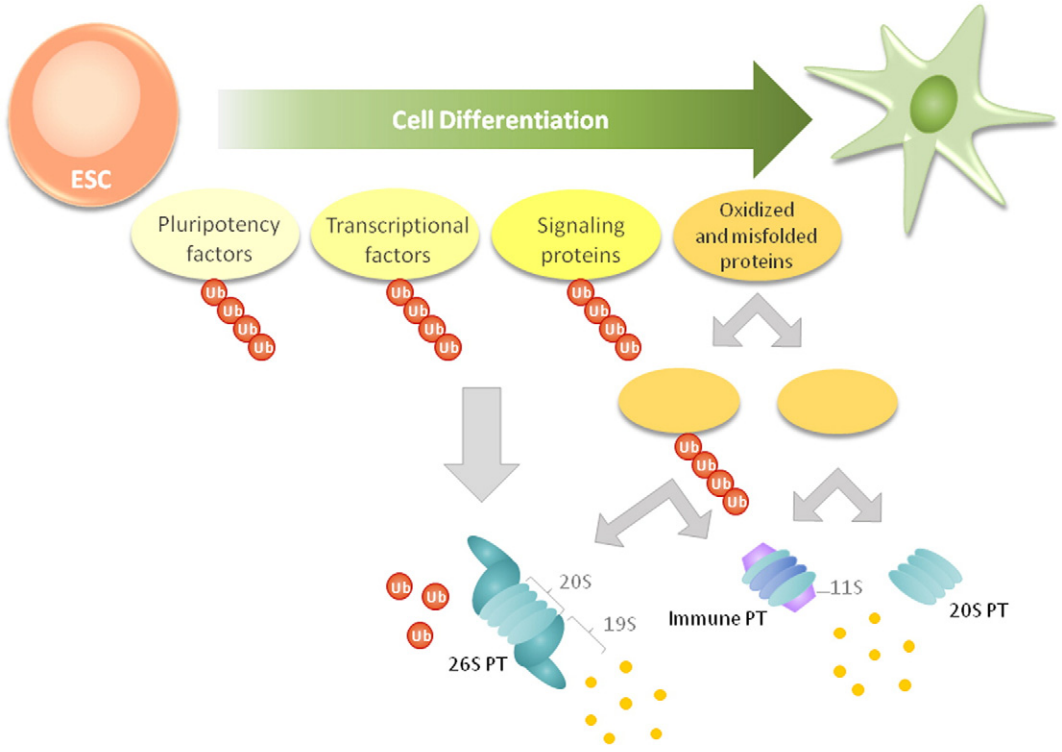

Fig. 1. Schematic representation of protein degradation in pluripotency and cellular differentiation. Proteins involved in the maintenance of pluripotency, transcriptional factors that trigger differentiation, and redox-modulated signaling proteins are regulated through poly-ubiquitylation, where the addition of at least four ubiquitin molecules are needed to induce their degradation by the 26S proteasome (20S catalytic particle coupled to 19S regulatory units). Alternatively, oxidized or misfolded proteins are degraded by the free 20S proteasomal pool or by the 20S-11S complex and 20S immune isoforms in cells undergoing differentiation, as previously described and discussed in the present review.

Table 1
E3 Ligases and their protein targets in cellular differentiation.

E3-Ligase	Target	Reference
SCF^{Fbxw7}	Notch	[80,81,83,92–96]
	c-Jun	[80,83,97]
	c-Myc	[77–79,82,98–100]
	C/EBPα	[101]
	OASIS	[102]
	BBF2H7	[102]
	KLF5	[103]
WWP1	Jun-B	[104]
	Runx2	[105]
	CXCR4	[106]
	Smad4	[107]
WWP2	Oct-4	[108–112]
Smurf	β-catenina	[113]
	Runx2	[86,87,89]
	Jun-B	[86,114]
	MEKK2	[88]
	Smad1	[86,89,115]
	Smad5	[116]
	TGF-β receptor	[117]
c-Cbl	STAT5	[90]
Itch	CXCR4	[118,119]
	Notch	[120]
Huwe1	N-myc	[121]
$SCF^{β\text{-}Trcp}$	REST	[122]
Mbi1	Notch	[123]
CHIP	Runx2	[124]
E6AP	C/EBPα	[125]
ASB4	ID2	[126]
Nedd4L	Smad2/3	[127]
Unknown	Nanog	[128,129]
	Osx	[130]

Protein mono-ubiquitylation, usually associated to gene expression, is critical for ESC maintenance [131,132]. Conversely, H2B mono-ubiquitylation was shown to increase during differentiation of stem and precursor cells [133,134].

3.2. Ubiquitylation induced by protein redox regulation

At the transcriptional level, cell differentiation is also regulated by the redox modification of transcription factors followed by their poly-ubiquitylation and consequent degradation. Examples are APE1/Ref-1, HIF, and Nrf2/Keap1, which function mainly as central regulators of the cellular response to redox shifts and other essential pathways of cell metabolism and have been associated with cell differentiation programs.

APE1/Ref-1 (Apurinic/apyrimidinic endonuclease 1/redox effector factor-1, referred here as APE1) is a protein involved in the DNA base-excision repair pathway and is a co-activator of some transcription factors that act as effectors of the oxidative stress response, such as NF-KB, HIF, and p53 [135]. APE1 is modulated by different post-translational modifications, such as phosphorylation, acetylation and ubiquitylation. Ubiquitylation of APE1 is regulated by the E3 ubiquitin ligases UBR3 and MDM2, depending on the cellular context [136–138]. APE1 participates in different cellular processes such as apoptosis, proliferation and differentiation [139]. Examples of cellular differentiation pathways regulated by APE1 are hematopoietic and neuronal differentiation of stem cells, both of which are dependent on the redox activity of APE1 but are independent of the DNA repair activity of APE1 [140,141]. During neuronal differentiation of human adult and embryonic stem cells, increased APE1-binding to chromatin is observed most likely because of the increased intracellular content of oxidants, which indicates an important redox function of APE1 in the neuronal differentiation process [141].

As already discussed above, oxygen availability in stem cell niches is an important factor that influences metabolism and differentiation. Hypoxia-inducible factors (HIFs) are transcription factors that sense and regulate adaptive responses to low O_2 tension. Under hypoxia, the HIF-α subunit (types 1, 2 or 3) is stabilized to form a heterodimer complex with the HIF-1β subunit. This complex then translocates to the nucleus and binds to hypoxia-responsive elements to activate genes encoding proteins that mediate processes related to hematopoiesis, angiogenesis and erythropoiesis, promoting cell survival [2,142–145]. Generally, hypoxia has been associated with enhanced stem cell pluripotency, while differentiation can be impaired or enhanced under hypoxia, depending on the cell type. HIF-1α and HIF-2α have been suggested to regulate stem cell function by altering Oct-4 and c-Myc expression, both of which are essential for stem cell multipotency [146,147]. HIF-1α is also involved in the Wnt/beta-catenin pathway under hypoxic conditions, which impairs cardiomyocyte differentiation in mouse iPS cells [148]. Under normoxic conditions, HIF-1α is rapidly degraded by the ubiquitin–proteasome system. HIF-1α is hydroxylated at a specific prolyl residue by prolyl hydroxylase that is recognized by a von Hippel–Lindau (VHL) protein that, in complex with Elongin-C and Elongin-B, functions as an E3-ligase, driving HIF-1α to proteasomal degradation [149]. The stability of HIF-1α can also be regulated by the E3 ubiquitin ligases CHIP and Hypoxia-Associated Factor [150,151].

The transcription factor Nrf2 is a key regulator of the antioxidant response and of enzymes in phase II of detoxification [152]. Under normal, unstressed conditions, Nrf2 is localized mainly in the cytoplasm where it is bound to the repressor Keap1 protein (Kelch-like ECH-associated protein 1), which targets Nrf2 for ubiquitylation and subsequent degradation by a Cul3 (cullin 3)-mediated ubiquitylation complex [153,154]. Keap-1 acts as a redox sensor. When Keap1 cysteines are oxidized, Nrf2 is released and migrates to the nucleus where it induces the expression of genes related to anti-oxidant defense and proteasome subunits as well as their assembly through the induction of the POMP chaperone, a proteasome maturation protein [153].

In stem cell differentiation, the Keap1-Nrf2 axis inhibits RANKL-induced osteoclast differentiation via ROS reduction, regulates the cell fate of HSCs, and promotes neuronal cell differentiation. [155–159]. Nrf2 was also shown to control self-renewal and pluripotency in hESCs by regulating proteasome expression [152].

3.3. Degradation of oxidized and misfolded proteins during differentiation

Although few reports in the literature have addressed the importance of proteostasis during differentiation to date, the fine regulation of malformed or oxidized protein clearance is expected. ERAD plays a fundamental role in the clearance of malformed proteins. ERAD is a UPS-dependent process [160]. On the other hand, the clearance of metabolically oxidized proteins is mainly supported by the free pool of the 20S core particle. As cells going through differentiation are under important pro-oxidative challenge, the pool of oxidized proteins is expected to increase. As discussed earlier in this review, mildly oxidized proteins are preferentially degraded by the 20S proteasome by a process independent of poly-ubiquitylation and ATP consumption. When cells go through an oxidative imbalance, one of the cellular defenses to maintain surveillance of the proteome is the unfolded protein response (UPR) to prevent the accumulation of misfolded proteins [161]. Quality control of mitochondrial proteins is achieved by chaperones and proteases, the so-called mitoUPR [162]. In the case of stem cells, reports in the literature have shown ESCs to have a high ability to respond to protein misfolding, as these cells have increased levels of heat-shock proteins when compared to differentiated cells [163]. Undifferentiated mouse ESCs were shown to contain high levels of protein carbonyls and the so-called AGE (advanced glycation end products), hallmarks of protein oxidation during the aging process [164]. Increased ability of cells to rid themselves of oxidized proteins upon differentiation was observed by comparing the content of oxidized proteins in the inner cell mass of blastocysts to that of cells differentiated to the

trophectoderm [164]. Notably, removal of oxidatively modified proteins during ESC differentiation was associated with increased expression of the proteasomal activator PA28 and of the immune proteasome subunits [165]. Upregulation of the immune 20S proteasome was also identified upon skeletal muscle differentiation and the knockdown of the immune proteasome catalytic subunit PSMB9 or the utilization of specific immune proteasome inhibitors, prevented differentiation of skeletal muscle cells [166]. Most interesting, suppression of the immune proteasome increased the pool of oxidized proteins and pro-apoptotic proteins. These results point to an increased oxidative environment during differentiation and to the important role of the 20S immune proteasomal variants on the removal of oxidized proteins. In conclusion, despite little data regarding the role of the 20S free pool during cell differentiation, conditions during differentiation appear to resemble those due to mildly oxidative stress.

4. Illustrating the role of UPS and redox regulation in pluripotency and differentiation of cardiomyocytes

In the present review, an interactome was built using systems biology by comparing ESCs with terminally differentiated cardiomyocytes (CM) of *Mus musculus* to illustrate the role of UPS and redox regulation during differentiation.

Data collected from the *M. musculus* interactome network (Supplementary Material) using proteins related to differentiation, the proteasome and mechanisms of redox homeostasis generated a network containing 282 nodes and 3771 connectors (Fig. 2). Major components regarding proteasome- and ubiquitin-dependent processes were identified, such as components of the 26S proteasome (PSMA, PSMB, PSMC and PSMD), ubiquitin-activating enzyme E1 (Uba1), ubiquitin-conjugating enzyme (UBE2C), and deubiquitylating enzymes (USP5; UCHL5). In addition, proteins associated with DNA repair (APEX1, RAD23A, RAD51; Table 2), anti-oxidant defense (PRDXs, CAT, SODs; Table 2) and development/cell differentiation (NOTCH1, SMAD3 and 4, among others; Table 2) were also observed in the interactome network (Fig. 2). By overlaying the interactome with RNA-sequence data and comparing ESCs with terminally differentiated cardiomyocytes (Supplementary Material), the following parameters were evaluated: (i) nodes under- and overexpressed in CMs compared to ESCs and (ii) major biologic processes transcriptionally regulated in CMs compared to ESCs (Figs. 3 and 4). Components of the cell cycle, ubiquitin-dependent processes, the stress response and anti-ROS defense (Table 2) were significantly underexpressed in CMs when compared to ESCs (Fig. 3).

Nodes representative of the UPS such as PSMD14, the deubiquitylating enzyme component of the proteasomal 19S regulatory particle as well as the E3 ligase SCF^{Fbxw7} were found to be underexpressed in CMs (Fig. 3). Notably, Psmd14 is a constitutive unit of the 26S proteasome, responsible for deubiquitylating substrates entering the catalytic chamber. PSMD14 has been shown to be a key component in the maintenance of ESC pluripotency [79]. Buckley and co-workers [79] have shown Psmd14 deubiquitylating activity as essential for pluripotency, as its overexpression inhibited differentiation. Moreover, these authors demonstrated that Psmd14-interacting proteins in the 19S lid are also important in the maintenance of pluripotency. Silencing Psmd11 and Psmd13,

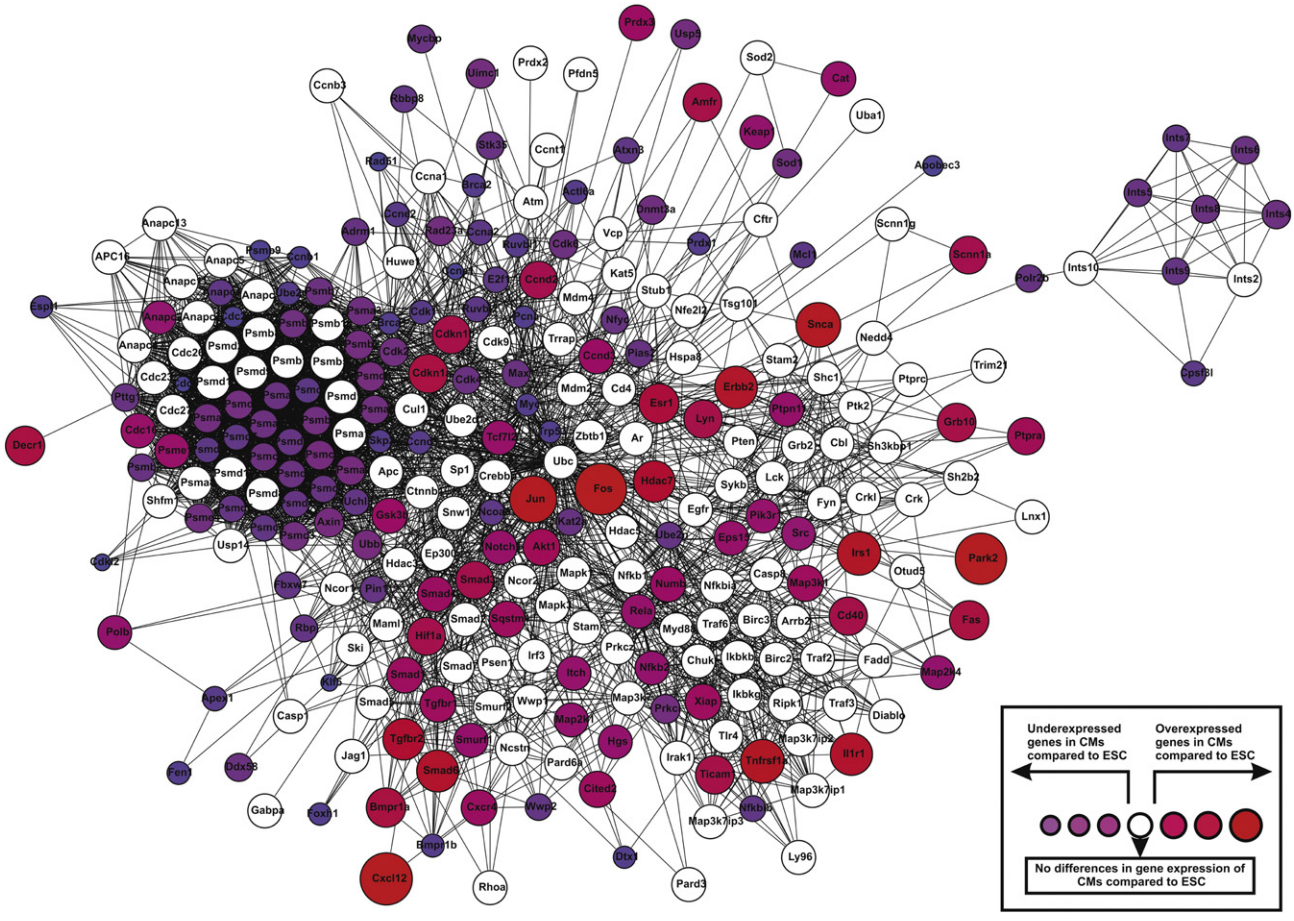

Fig. 2. The major interactome network obtained from proteins related to UPS, cell differentiation and redox mechanisms. Data on differentially expressed genes (DEGs) generated from RNA-seq analysis of cardiomyocytes (CMs) and embryonic stem cells (ESC) were overlaid in the network. Overexpressed nodes in CMs compared to ESCs are displayed in larger node sizes and red colors (inset), while underexpressed nodes in CMs compared to ESCs are shown in smaller node sizes and blue colors (inset). Nodes that did not displayed gene expression differences are indicated in white (inset).

Table 2
Specific gene ontology (GO) classes derived from under- and overexpressed nodes selected from the interactome network associated to proteasome, cell differentiation and redox mechanisms.

Node state	GO biological process category	GO number	*p*-value	Corrected *p*-value[a]	k[b]	f[c]	Gene
Underexpressed	Cell cycle	7049	1.48×10^{-18}	1.01×10^{-16}	23	613	TRP53;E2F1;CDC6;CDK1;SKP2;BRCA2; CDC20;ANAPC10;ESPL1;CDK6; PTTG1; CDK4;UBE2C;BRCA1;CDK2;RAD51; RBBP8;CCNB1;CCNE2; CCNE1;CCND1; RUVBL1;CCNA2
	UPS	6511	5.99×10^{-15}	3.26×10^{-13}	12	124	UBE2N;PSMA2;PSMA6;PSMA5;WWP2; RAD23A;USP5;PSMA4;PSMA3; UCHL5;SKP2;PSMA7
	DNA repair	6281	1.44×10^{-14}	6.52×10^{-13}	14	225	TRP53;RAD23A;BRCA2;PTTG1;SOD1;UIMC1;BRCA1;RAD51;UBE2N; PCNA;PSMD4;RUVBL2;APEX1;FEN1
	Response to DNA damage	6974	2.22×10^{-14}	9.67×10^{-13}	15	289	TRP53;CDK1;RAD23A;BRCA2;PTTG1;SOD1;UIMC1;BRCA1;RAD51; UBE2N;PCNA; PSMD4;RUVBL2;APEX1;FEN1
	Response to stress	6950	1.70×10^{-9}	3.19×10^{-8}	20	1243	TRP53;CDK1;RAD23A;BRCA2;PTTG1;SOD1;PRDX1;UIMC1;BRCA1; RAD51;DDX58; UBE2N;CCND1;PCNA;PSMD4;RUVBL2; RBPJ;BMPR1B; APEX1;FEN1
	Cellular response to reactive oxygen species	34,614	3.96×10^{-5}	3.39×10^{-4}	3	21	SOD1;APEX1;PRDX1
	Cellular response to superoxide	71,451	1.48×10^{-4}	1.02×10^{-3}	2	6	SOD1;PRDX1
Overexpressed	Organ development	48,513	2.31×10^{-18}	2.64×10^{-16}	23	1680	LYN;ERBB2;TGFBR1;TGFBR2;SMAD4;ESR1;SMAD3;SMAD1;PRDX3;IRS1; CXCL12; CITED2;AKT1;HIF1A;CDKN1B;CXCR4; GSK3B;JUN;MAP3K1; NUMB;FAS;HDAC7; BMPR1A
	Positive regulation of cell proliferation	8284	2.63×10^{-18}	2.79×10^{-16}	15	386	LYN;ERBB2;TGFBR2;ESR1;CD40;CXCL12; IRS1;CDKN1A;CDKN1B;HIF1A; CCND3; CCND2;JUN;TICAM1;BMPR1A
	Cell differentiation	30,154	5.72×10^{-17}	4.04×10^{-15}	22	1697	LYN;ERBB2;TGFBR1;PTPRA;TGFBR2; SMAD4;ESR1;SMAD3;SMAD1; PRDX3; CXCL12;CITED2;AKT1;HIF1A;CXCR4; SQSTM1;GSK3B;JUN; NUMB;FAS;HDAC7; BMPR1A
	Immunological process	2376	6.75×10^{-16}	2.94×10^{-14}	16	700	IL1R1;LYN;TGFBR1;SNCA;TGFBR2;SMAD3;CD40;PRDX3;CXCL12;AKT1; HIF1A;CCND3;CXCR4;TICAM1;FAS;HDAC7
	Blood vessel development	1568	1.06×10^{-10}	2.29×10^{-9}	9	265	AKT1;HIF1A;CXCR4;JUN;TGFBR1;TGFBR2;CXCL12;HDAC7;CITED2
	Heart development	7507	9.04×10^{-7}	7.10×10^{-6}	6	233	HIF1A;TGFBR1;ERBB2;TGFBR2;SMAD3;BMPR1A

[a] Values calculated from *p*-value after FDR application.
[b] Total number of genes found in the network that belong to a specific GO.
[c] Total number of genes belonging to a specific GO.

both Psmd14-interacting subunits, induced differentiation. Other components of the proteasome structure also appear to be significantly downregulated in the interactome network (Figs. 1 and 2), e.g., PSMC5 and PSMD7. Based on these data, the UPS seems to be essential for pluripotency. Accordingly, downregulation of the E3 protein SCF^{Fbxw7} was shown to control c-Myc protein stability to maintain the pluripotent state of ESCs [76]. These data highlight the importance of UPS integrity and functionality as a key system to maintain the pluripotent state.

Regarding the 20S proteasome specifically, the node represented by PSMB9 (Fig. 3), a component of the immune proteasome that is shown to be underexpressed in CMs, was found to be essential for murine and human skeletal myoblast differentiation, as PSMB9 knockdown or specific immune proteasome inhibition prevented differentiation in the murine and human cellular models, respectively [166]. As discussed above (Section 3.3), the immune proteasome was found to be essential for the removal of oxidized proteins during differentiation.

In recent years, a growing set of information has identified ubiquitylation (formation of mono- or poly-ubiquitin chains) as an essential mechanism to accomplish the repair of various types of endogenous and exogenous DNA damage. Proteins directly involved in the ubiquitylation process itself form protein complexes that are required for recovery from DNA damage, including protein targets for ubiquitylation containing ubiquitin-binding domains and deubiquitylating enzymes [167]. As depicted in Figs. 2 and 3 and Table 2, many of the genes found to be downregulated in terminally differentiated cells are related to DNA repair and response to DNA damage stimulus. The components of nucleotide and base excision repair (NER and BER, respectively) such as APEX1 and FEN1 as well as some members of the recombination pathway (e.g., RAD51, BRCA1, BRCA2) display significant low gene expression in CMs when compared to ESCs. Both APEX1/REF1 and FEN1 are components of the BER pathway and are responsible for the removal of damaged bases from DNA, especially those that originated from the oxidative processes [168]. Apex1 is an apurinic/apyrimidic endodeoxyribonuclease whose inhibition by small interfering RNA in cardiac stem cells was shown to induce extensive apoptosis and cardiomyocyte differentiation [169]. On the other hand, FEN1, a 5′-flap endonuclease necessary for the repair of DNA damage induced by H_2O_2 [170], does not have a defined role in the maintenance of pluripotency. Similarly to APEX1, the expression of FEN1 in ESCs most likely impairs the promotion of cell differentiation. A potential mechanism where FEN1 may block cell differentiation might be related to the regulation/control of the cell cycle, which is necessary for the maintenance of ESC self-renewal. In fact, high FEN1 protein levels are directly connected to the S phase of the cell cycle, as FEN1 is further ubiquitylated and degraded by the proteasome after cells exit S phase [171]. Considering that ESCs display a long S phase [172], FEN1 most likely acts during S phase to promote genome, and consequently, the pluripotency of ESCs is maintained. The CDC6 gene, whose product is necessary for initiation of DNA replication and promotion of S phase, was shown to be significantly downregulated in cardiomyocytes when compared to ESCs (Figs. 2 and 3). This finding supports the idea that maintenance of the cell cycle and, specifically, DNA replication and S phase, is essential to avoid cell differentiation.

Components of redox homeostasis, e.g., peroxiredoxin 1 (PRDX1) and, to a lesser extent, superoxide dismutase (SOD1), were found to be downregulated in CMs when compared to ESCs (Figs. 2 and 3). The role of both proteins in the self-renewal/pluripotency of ESCs is not clear. However, according to the interactome network (Fig. 2), a connection between PRDX1 and ADRM1 is depicted. Adrm1 is a proteasomal 19S regulatory unit-associated protein that recruits ubiquitin carboxyl-terminal hydrolase L5 (UCHL5; Fig. 2), a deubiquitylating enzyme and a putative regulatory component of the chromatin remodeling complex INO80 [173]. Experimental data have pointed to an intricate cooperation among the INO80 complex, ADRM1 and UCHL5 in

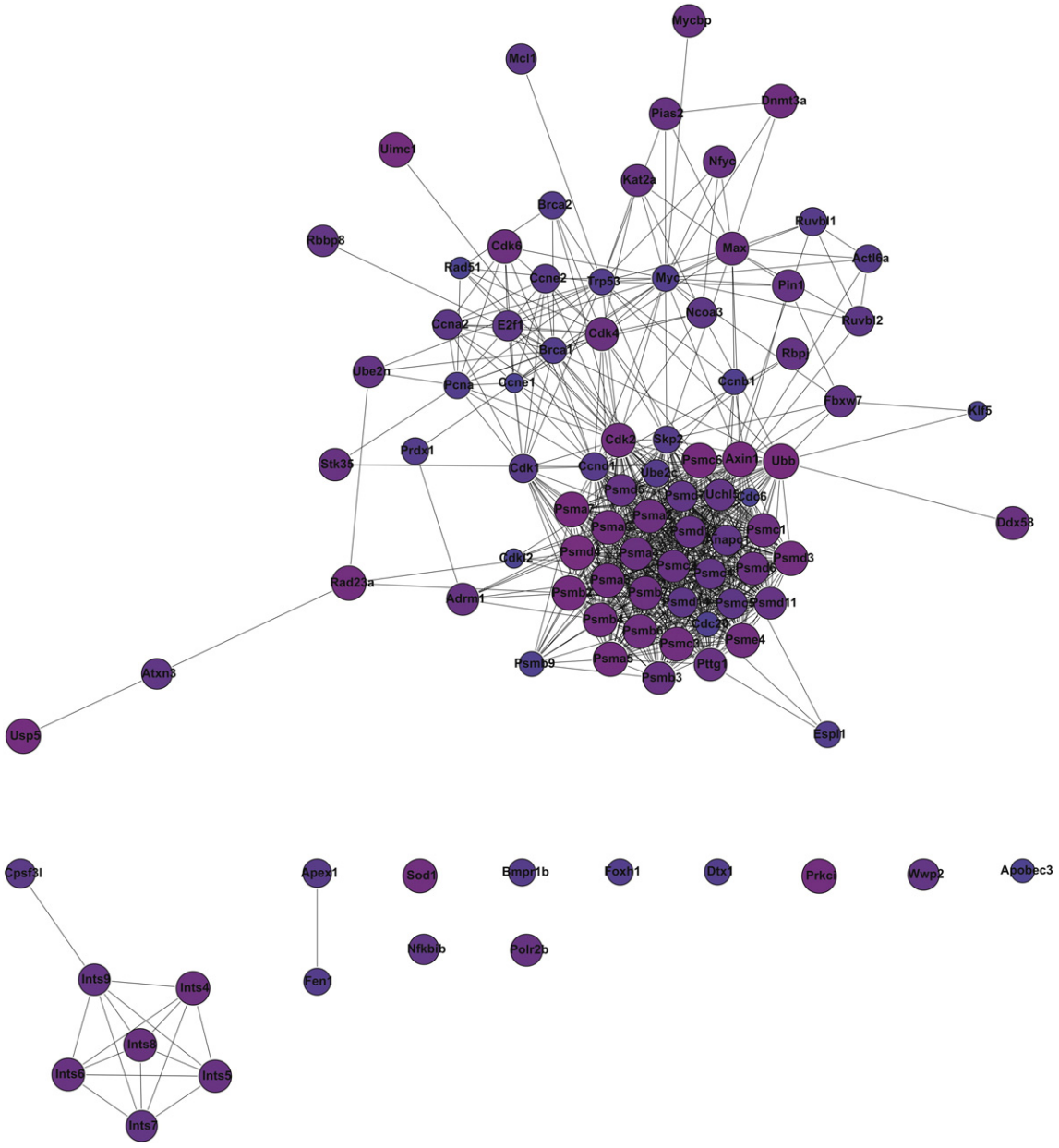

Fig. 3. Underexpressed clusters and nodes observed in cardiomyocytes (CMs) when compared to embryonic stem cells (ESCs) as gathered from RNA-seq data analysis. The clusters and nodes indicated in this figure were selected from the major interactome network of proteins related to UPS, cell differentiation and redox mechanisms.

chromatin remodeling/gene expression and DNA repair [174]. Notably, the INO80 complex is necessary for ESC self-renewal/pluripotency [175]. This connection allows the hypothesis that PRDX1 is a redox sensor and thus a potential regulator of INO80 in ESCs.

Finally, data regarding the overexpressed genes in CMs compared to ESCs (Figs. 2 and 4; Table 2) indicate major biological processes related to organ, blood vessel and heart development, as they contain some overexpressed nodes already known to participate in stem cell differentiation, such as JUN and FOS, both of which are part of the AP-1 complex [176], and SMADs, a family of signal transduction genes activated in response to TGF-β signaling [177]. However, some of the overexpressed genes are components of immune processes, e.g., CXCL2, a significantly overexpressed node (Figs. 2 and 4). CXCL2 encodes a stromal cell-derived α-chemokine, a member of the intercrine family whose product has been implicated in embryonic development [178]. Recently, Law and co-workers [178] demonstrated that, through an in vitro approach by inducing CM differentiation from ESCs, ROS signaling was associated with the modulation of the AP-1 complex and cytokine synthesis as part of the mechanism to enhance cardiomyogenesis from ESCs, suggesting the interplay of immune components with redox modulation. Additionally, the peroxiredoxin 3 node (PRDX3; Figs. 2 and 4) is likely part of the immune and differentiation processes (Table 1). PRDX3 has been implicated in proerythrocyte induction [179], but its role in ESC differentiation is unknown.

5. Concluding remarks

Pluripotency, self-renewal and differentiation in several cellular models (ESC, HSC and other stem cell lineages) are a matter of intense investigation, as comprehension of these processes implies a potential therapeutic application in many human diseases. Redox regulatory processes and UPS play important roles in the central steps of either pluripotency or differentiation. As noted in the present review, stem cells reside into low O_2 niches. However, a wide range of O_2 concentrations allows for

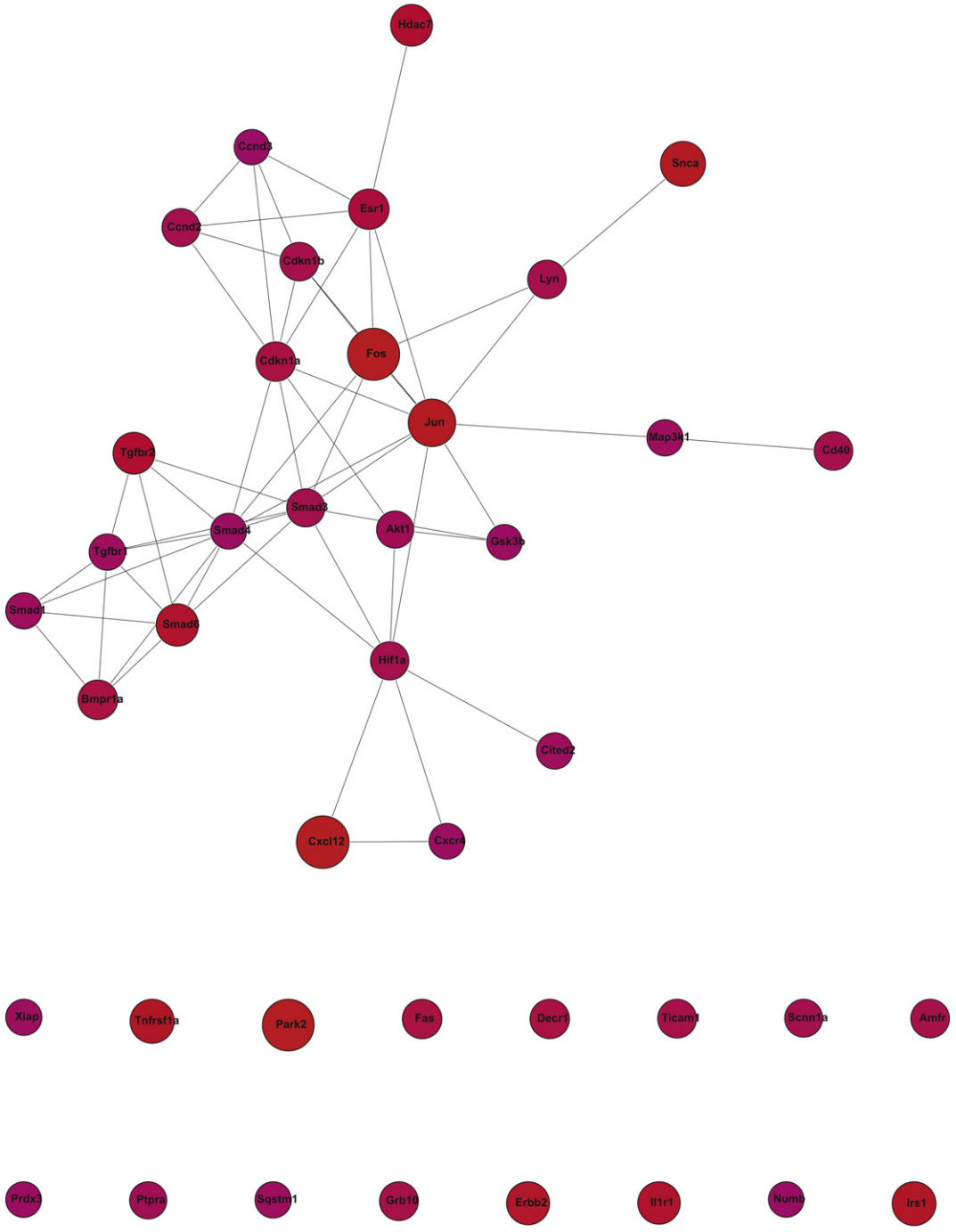

Fig. 4. Overexpressed cluster and nodes observed in cardiomyocytes (CMs) when compared to embryonic stem cells (ESCs) as gathered from RNA-seq data analysis. The cluster and nodes indicated in this figure were selected from the major interactome network of proteins related to UPS, cell differentiation and redox mechanisms.

differentiation resulting in the modulation of cell fate. In any condition, the ROS concentration is increased during differentiation, which most likely determines shifts in the activity of cellular effectors, such as kinases, phosphatases, and transcription factors, to trigger further steps of the process.

According to the data retrieved from the specific interactome network of cardiomyocytes differentiated from *M. musculus* ESCs, the role of UPS in the maintenance of pluripotency is noteworthy. Likewise, genome stability is likely essential for the pluripotency state. On the other hand, as cells differentiate, genes related to development and immune modulation prevail as shown by the overexpression of genes relying on both processes. Remarkably, the latter set of genes was found to be intimately related to redox modulation, as the PRDX3 node is linked to the immune response of differentiated cells.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.10.031.

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