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Beyond keratinocyte differentiation: emerging new biology of small proline-rich proteins

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Small proline-rich proteins (SPRRPs) are traditionally known for their function in keratinocyte homeostasis. Recent evidence demonstrates their involvement in additional diverse physiological processes ranging from p53 signaling and direct prevention of DNA damage to bactericidal activities. We highlight these novel, intriguing roles of SPRRPs and discuss them in the context of relevant pathological conditions.

SPRRPs exhibit exceptional functional versatility and biological pleiotropy

The multigene family of SPRRPs consists of hitherto identified 11 structurally highly homologous members: SPRR1A, SPRR1B, SPRR2A-G, SPRR3, and SPRR4. One of the most extensively studied biological functions of SPRRPs is their role in the formation of the **cornified cell envelope (CE)** (see Glossary) in **keratinocytes.** SPRRPs' activity becomes vital during later phases of keratinocyte differentiation, when their glutamine- and lysine-rich N and C termini are cross-linked with loricrin by calcium-dependent transglutaminases 1 and 3, resulting in the formation of an insoluble, highly protective CE (Figure 1A). SPRRPs dictate the CE's toughness, strength, and flexibility, characteristic of stratified squamous epithelia [1]. The property that sets SPRRPs apart from other CE components is their highly repetitive proline-rich central domain, characterized by a variable consensus sequence between the different family members.

However, recent findings suggest that SPRRPs are involved in additional pleiotropic biological functions that go beyond their established role in keratinocyte homeostasis. SPRRPs have been shown to facilitate cell migration and wound healing in keratinocytes [2], induce **epithelial–mesenchymal transition** in cholangiocytes [3,4], and alter invasiveness and metastatic capabilities in cholangiocarcinoma models [4] (Figure 1B). Intriguingly, SPRRPs play important roles in free radical quenching [2,5–7], direct prevention of chromosomal damage [5], and **p53** signaling [3,8], linking them to the pathogenesis of many diseases, including fibrosis-associated heart failure and cancer [8–10]. A testament to the family's remarkable versatility is the recent identification of SPRR2A as a novel antimicrobial protein capable of selectively killing Gram-positive gut bacteria by disrupting their cell membranes [11]. Similarly, SPRR1A and SPRR2A are active against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*, thus supporting the formation of cutaneous barrier that defends the host against systemic infection [12].

SPRRPs protect cells against free radicals and DNA damage

In addition to providing protection against mechanical, chemical, and biological noxae as part of the CE in squamous tissues, SPRRPs can directly quench **reactive oxygen species (ROS)** via oxidation of cysteine residues abundant in their primary structures (Figure 1C). The importance of cysteine in protection from oxidative stress was confirmed through chemical modifications of its residues, which resulted in an almost complete loss of SPRR1B's ROS-quenching capacity [2,5]. Cysteine oxidation triggers the formation of SPRRP multimers, whereby SPRRPs spontaneously associate into dimers, trimers, and tetramers via the formation of cysteine **disulfide bonds**, thus giving a rise to a large cytoplasmic structure referred to as a cellular antioxidant shield [5].

Furthermore, SPRRPs bind directly to DNA to prevent strand breaks upon exposure to ROS. Both quenching of ROS via cysteine oxidation and protection from DNA strand breaks via direct DNA binding are tightly regulated by the oxidation state of SPRRPs [5]. Although members of all four classes of SPRRPs possess the ability to bind DNA, SPRR4 required ten times

Glossary

Acetylation: a post-translational modification of proteins whereby an acetyl group is introduced onto a lysine residue, affecting its biochemical properties.
Biomarker: a naturally occurring molecule, gene, or characteristic by which a particular pathological or physiological process or disease can be identified.
Cornified cell envelope (CE): an insoluble structure, 15 nm thick, formed beneath the plasma membrane of epithelial cells and essential for providing protection against extracellular and environmental factors.
Deacetylation: the removal of acetyl groups from proteins.
Disulfide bond: a covalent bond formed under oxidizing conditions between the thiol groups of cysteine residues in proteins.
Epithelial–mesenchymal transition: a process by which epithelial cells lose their cell polarity and cell–cell adhesion, and gain migratory and invasive properties.
Helminth: a member of a group of multicellular eukaryotes, mostly consisting of parasitic worms.
Keratinocyte: epithelial cell found in the epidermis, the outermost layer of the skin.
Lipopolysaccharide (LPS): component of the cell wall found in the outer membrane of Gram-negative bacteria consisting of a lipid and a polysaccharide.
MDM2: a critical negative regulator of the tumor suppressor p53; it plays a key role in controlling p53 transcriptional activity, protein stability, and nuclear localization.
p53: a protein whose expression is induced by DNA damage; it acts as a tumor suppressor by stimulating cell-cycle arrest, DNA repair, cellular senescence, or apoptosis.
Reactive oxygen species (ROS): atoms or molecules that have one or more unpaired valence electrons, which makes them chemically reactive.
Ubiquitination: a post-translational modification in which a ubiquitin residue is attached to a substrate protein, generally tagging the protein for degradation via the proteasome degradation pathway.

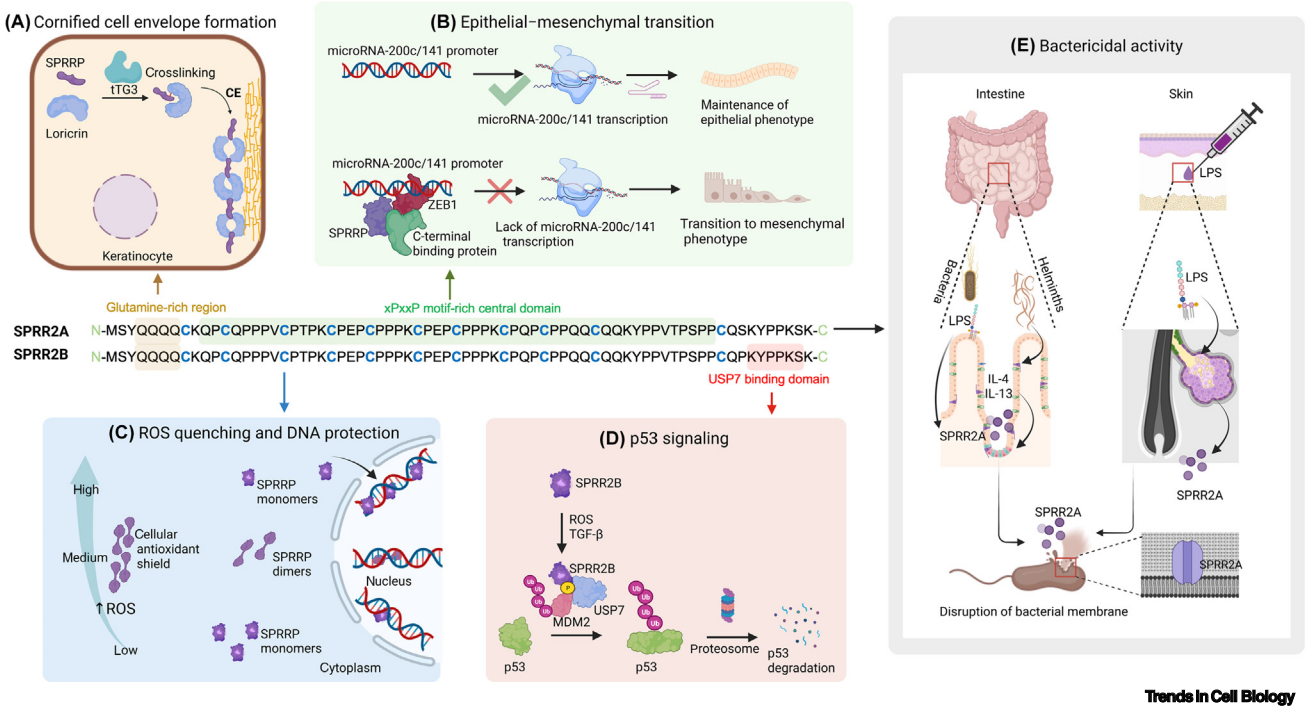

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Figure 1. Pleiotropic biological functions of small proline-rich proteins (SPRRPs): the examples of SPRR2A and SPRR2B. (A) SPRRPs as constituents of cornified cell envelope (CE). During later stages of keratinocyte differentiation, SPRRPs become a substrate for calcium-dependent tissue transglutaminase 3 (tTG3), which cross-links their glutamine-rich N termini with loricrin. This results in the formation of an insoluble, highly protective CE, characteristic of mature keratinocytes. (B) SPRRP expression increases invasiveness and induction of epithelial–mesenchymal transition (EMT). SPRRPs complex with ZEB1 and C-terminal binding proteins (CTBPs) on the microRNA-200c/141 promoter, leading to suppression of microRNA-200c/141 transcription and consequent induction of EMT. Critical for the initiation of these interactions are the specific proline-containing motifs within the central domain of SPRRPs, the minimal one being xPxxP. (C) SPRRPs in protection against reactive oxygen species (ROS) and DNA damage. At lower ROS levels, SPRRPs are distributed within the cell globally; the cytoplasmic fraction quenches ROS, while the nuclear fraction associates with DNA. Increase in ROS levels leads to oxidation of cysteine residues and SPRRP multimerization, which allows them to build a cellular antioxidant shield in the cytoplasm. At even higher ROS levels, the disulfide bonds are broken, the antioxidant shield dissolves, and the SPRRP monomers preferentially localize to the nucleus to protect the DNA from chromosomal damage. (D) SPRRPs in p53 signaling pathway. Transforming growth factor β (TGF-β) and ROS lead to phosphorylation of the Y-67 residue of SPRR2B, resulting in SPRR2B binding with ubiquitin-specific protease 7 (USP7) and mouse double minute 2 homolog (MDM2). Formation of the SPRR2B–USP7–MDM2 complex stimulates increased removal of ubiquitin residues from MDM2 by USP7, thereby stabilizing MDM2 and allowing its continuing interaction with p53. p53 is consequently ubiquitinated and degraded. (E) SPRRPs act as antimicrobial proteins. Exposure to the bacterial cell wall component lipopolysaccharide (LPS) triggers SPRR2A expression in the goblet and Paneth cells in the intestine and in the sebaceous gland cells in the skin. SPRR2A then disrupts the bacterial membrane through binding to negatively charged lipids, killing the bacteria. Gut exposure to helminths elicits a similar response via type 2 cytokines interleukin-4 (IL-4) and IL-13, which also induce SPRR2A expression, thus preventing bacterial invasion due to the helminth-induced weakening of the gut wall. Abbreviations: tTG3, tissue transglutaminase 3; ZEB1, zinc finger E-box binding homeobox 1.

lower molar quantities to achieve comparable DNA binding relative to SPRR1-3 class proteins [5].

The importance of SPRRPs in defense against ROS in renal cells was demonstrated in a ureteral obstruction mouse model, where SPRR2F was among the most upregulated genes following renal injury. SPRR2F-knockout mice exhibited greater renal damage following unilateral ureteral obstruction compared to wild-type animals. This was validated by a significant decrease in cell viability upon ROS induction in renal epithelial cells derived from SPRR2F-knockout animals, which could be rescued by glutathione supplementation [6].

Similar protective effects were observed in cardiac cells. *In vitro* models of ischemic stress featured less structural damage and markedly reduced rates of apoptosis in rat cardiomyocytes overexpressing SPRR1A. A significantly higher tolerance to ischemic stress and absence of markers related to global cardiac injury were confirmed *in vivo* in SPRR1A-overexpressing murine cardiomyocytes. In these models, SPRR1A accumulated along myofibrils, where it engaged in ROS quenching to protect cardiomyocytes from ROS-mediated damage [7].

SPRRPs in the p53 signaling pathway

The observation that some processes stimulated by SPRR2A overexpression – such as increased cell viability upon ROS

exposure, and acquisition of mesenchymal properties – may be impeded by p53-induced cell senescence or death under harmful conditions led to the hypothesis that SPRR2A may need to suppress p53 activity to exert these effects. Indeed, it has since been shown [3] that SPRR2A affects p53 stability through its **deacetylation**. This is accomplished via two distinct pathways: first, SPRR2A interferes with p300–p53 binding and diminishes p300-mediated transfer of acetyl groups onto p53 by complexing with the CH_3 domain of p300 through its xPxxP motifs, and second, SPRR2A increases expression of histone deacetylase 1 (HDAC1), resulting in direct HDAC1-mediated removal of acetyl groups from p53. Additionally, by competing with p53 for binding to p300, HDAC1 can interfere with p300-mediated p53 **acetylation**.

K382 acetylation of p53 stabilizes the protein and leads to its accumulation, allowing p53 to exert its transcriptional activity on many cellular promoters and leading, for example, to enhanced p21 expression [3]. p21 promoter activity is significantly reduced in SPRR2A-overexpressing cholangiocarcinoma cells, the decrease in p53 acetylation being related to a reduction in expression of its downstream effectors [3].

Interestingly, a separate mechanism that links one of the SPRRPs to p53 stability has been described recently [8] (Figure 1D). In a mouse model of congestive heart failure (CHF), SPRR2B expression activated fibroblasts and increased their proliferation, specifically upon exposure to transforming growth factor β and oxidative stress, two of the known stimuli of cardiac pathological remodeling. The underlying process proved to be SPRR2B binding to ubiquitin-specific protease 7 (USP7), a ubiquitin ligase, which interacts with and deubiquitinates mouse double minute 2 homolog (**MDM2**). Formation of the SPRR2B–USP7 complex leads to increased removal of ubiquitin residues from MDM2, which allows MDM2 interaction with p53 and consequently promotes its **ubiquitination** and degradation. In CHF, these events attenuate apoptosis and increase the expression of several cyclins, such as CDK1, CCA2K or CCNB1, thus allowing cell-cycle progression [8]. The SPRR2B-USP7 interaction is strongly induced by SPRR2B tyrosine-67 phosphorylation, presumably by the SRC nonreceptor tyrosine kinase [8]. Although direct involvement in MDM2–p53 signaling pathway has so far been reported only for SPRR2B, it is likely that, due to the high degree of homology and possession of the seemingly critical tyrosine-67 residue, other class members such as SPRR2A and SPRR2G can engage in analogous interactions.

SPRRPs as cancer biomarkers

The engagement of SPRRPs in p53 signaling and in protection from DNA damage foreshadows their implication in tumorigenesis and in responses to DNA damage-based anticancer treatments. Indeed, SPRRPs are increasingly being linked to various human neoplasms. Of clinical interest are the findings indicating SPRRPs as plausible cancer **biomarkers**. Median serum SPRR2A concentration in gastric carcinoma (GC) patients was significantly higher than in healthy controls, exhibiting 75.7% sensitivity and 74.5% specificity [9]. SPRR2A serum levels in GC patients were significantly associated with lymph-node metastasis and the tumor–node–metastasis (TNM) stage ($P < 0.05$).

In a similar fashion, SPRR2A was one of the most consistently downregulated transcripts in metastatic and recurrent cells in a panel encompassing patient-matched primary and secondary head-and-neck squamous-cell carcinoma (HNSCC) models [10]. Furthermore, SPRR2A was downregulated in HNSCC primary tumors (61.9% of cases) and lymph-node metastases (31.3%), but not in normal tissue. High expression of SPRR2A in lymph-node metastases was identified as an independent prognostic factor for regional disease recurrence after surgery and radiotherapy [10]. These findings are likely to pave the road for exploration of additional features that relate SPRRPs to the onset, maintenance, and progression of malignant disorders.

SPRRPs possess bactericidal properties and act as antimicrobial proteins

The first hint of a role for SPRRPs in antimicrobial defense was the observation that colonization of a germ-free mouse intestine with the commensal *Bacteroides thetaiotaomicron* markedly increases expression of mouse SPRR2A [11]. SPRR2A expression was triggered by exposure to the bacterial cell wall component **lipopolysaccharide (LPS)**, mediated through TLR4-MyD88 signaling and mostly restricted to goblet and Paneth cells of the intestinal epithelium, from which SPRR2A was secreted in the intestinal lumen [11]. SPRR2A, which has under physiological conditions basic pH, subsequently interacted with the bacterial membrane by binding to lipids bearing negatively charged headgroups, thus disrupting bacterial membranes and eliciting a dose-dependent reduction in the viability of the Gram-positive species *Listeria monocytogenes*, *Enterococcus faecalis*, and *Lactobacillus reuteri* (Figure 1E). Interestingly, despite the ability of LPS to induce SPRR2A expression, Gram-negative bacteria were unaffected by this phenomenon as LPS neutralized SPRR2A-mediated membrane permeabilization [11].

SPRR2A also acts in antibacterial defense during **helminth** infections (e.g., by *Heligmosomoides polygyrus*), which can promote bacterial overgrowth and breach the intestinal barrier. This is mediated via type 2 cytokines interleukin 4 and interleukin 13, both secreted in response to helminth infection and stimulating SPRR2A expression. Compellingly, the helminth-induced protection against

bacterial invasion makes SPRR2A unique among previously described antimicrobial proteins [11].

Analogous findings were reported for other SPRRP family members, which form an integral part of the cutaneous barrier in protection against skin infections [12]. Both LPS exposure of human immortalized sebaceous gland cells and LPS injection into the skin of germ-free mice resulted in a significant MYD88-mediated increase in mouse SPRR1A and SPRR2A expression (Figure 1E). Mouse as well as human SPRRPs displayed potent activity against a wide range of bacteria – including MRSA and *P. aeruginosa* (one of the most common causes of mortality in burn patients). SPRRPs bactericidal activity was equivalent to the previously described bacterial membrane disruption in intestinal bacteria [12], and these effects were replicated *in vivo* where Sprr1a$^{-/-}$ and Sprr2a$^{-/-}$ mice were more susceptible to MRSA and *P. aeruginosa* infections, indicating their integral role in the formation of a cutaneous barrier defending the host against systemic infection.

Concluding remarks

Harmful processes such as inflammation and oxidative stress, both of which are known to increase SPRRPs expression, require rapid and efficient adaptation to maintain cellular survival. We highlight SPRRPs as essential components of the epithelial tissue defense machinery under such stressful conditions. SPRRPs quench ROS and directly protect cells from chromosomal damage, with consequent effects on genome stability through DNA binding. Complementarily, SPRRPs downregulate p53 expression by diminishing its acetylation and enhanced ubiquitination, thus making cell-cycle arrest and apoptosis less likely. This allows SPRRPs to act as active mediators in the cellular DNA damage response and implicates them in the pathophysiology of CHF, acute kidney injury, and cancer. Additionally, SPRRPs expression can alter cellular phenotype by inducing migratory capabilities and epithelial–mesenchymal transition, thus allowing rapid cell mobilization under physiological conditions, or affecting chemoradioresistance in pathological circumstances such as cancer. Furthermore, SPRRPs guard the host via bacterial membrane disruption, thereby providing additional protection against bacterial invasion. In the era of rapidly emerging multidrug-resistant bacterial strains and the worrying shortage of novel antibacterial therapies, these exciting new findings may offer next-generation avenues to tackle frequently lethal pathogens, such as MRSA and *P. aeruginosa*.

A major challenge in the SPRRPs field is represented by the high homology between SPRRPs, which in some cases differ only in one amino acid. This presumably leads to a high degree of biological redundancy and the capability of stringent regulation through a tight differential tissue/cell-type expression. Consequently, various members of the family may carry out analogous functions in different tissues and fine-tune cellular response to different or specific stimuli (e.g., induction of SPRR2 family members in response to UV light in keratinocytes, induction of SPRR2B in response to ROS exposure in cardiac fibroblasts, or induction of SPRR2A in response to LPS in intestinal goblet cells). Conversely, other biological functions, such as DNA binding by SPRR4 or direct engagement in MDM2-p53 signaling by Y67-bearing SPRR2 proteins, appear to be restricted to one class or certain class members and dictated by minor but relevant alterations in protein primary structure. Further research is necessary to decipher these fine nuances and gain a complete understanding of this captivating family of proteins and their physiologic regulation.

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Declaration of interests

There are no conflicts of interest to disclose.

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