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Review

From mammals to viruses: the *Schlafen* genes in developmental, proliferative and immune processes

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Abstract

The *Schlafen* genes have been associated with proliferation control and with several differentiation processes, as well as with disparate phenotypes such as immune response, embryonic lethality and meiotic drive. They constitute a gene family with widespread distribution in mammals, where they are expressed in several tissues, predominantly those of the immune system. Moreover, horizontal transfer of these genes to orthopoxviruses suggests a role of the viral *Schlafens* in evasion to the host immune response. The expression and functional studies of this gene family will be reviewed under the prism of their evolution and diversification, the challenges they pose and the future avenues of research.

Keywords: copy number variation; DDK syndrome; differentiation; gene family; horizontal transfer; immune response; meiotic drive; poxvirus; proliferation; redundancy; *Schlafen*.

Introduction

The *Schlafen* (*Slfn*) gene family was first identified in mouse during a screening for genes involved in thymocyte maturation (1). Since then, they have been found not only in placental mammals, but also in marsupials as well as other vertebrates, and even in viruses (1–3). *Slfn* gene products have been associated with a variety of processes that include proliferation and differentiation of diverse cell types, especially those of the immune system. During the last few years, new findings about their functional similarities and particularities have substantially increased the interest for this gene family.

Slfn1-4 were the first members of this family identified in mouse (Figure 1) by Schwarz et al. (1). The other two members described by these authors, *Slfn6* and *Slfn7*, were probably *Slfn3* and *Slfn4* isoforms [or other homologous mouse sequences (paralogues)]; in any case, these terms no longer correspond to any known gene, although they still create some confusion. Subsequently, *Slfn5*, *Slfn8*, *Slfn9* and *Slfn10* were discovered in mouse (4) (Figure 1). In addition, other paralogous sequences (*LOC435271* and *Slfn14*) have been identified (2, 3, 5) (Figure 2). The SLFN1-10 proteins display a strong similarity (51%–96%) over a common shared region (3) (Figure 1). Although they contain several conserved domains (such as a divergent AAA domain), no significant similarity has been found with other mammalian genes. Only a sequence annotated as *Slfn-like 1* on mouse chromosome 4 codes for a peptide with very low similarity to part of the SLFN AAA_4 domain. Therefore, it is not considered a 'bona-fide' *Slfn* family member (3).

The study of the function of the *Slfn* genes has encountered three major difficulties, which are common in gene families studies. The first one has been posed by the close sequence similarity of the *Slfn* family members, especially among some of them (e.g., mouse *Slfn3* and *Slfn4*, or *Slfn8-10*). This similarity, at both the nucleotide and the amino acid level, has hampered the design of gene (protein)-specific experimental tools (such as primers, probes and antibodies) and the interpretation of the results. Therefore, characterization of the expression and function of each *Slfn* member must be always accompanied by a solid validation of the identity of the gene sequences/products under analysis, in order to avoid mixed and confounding results among the paralogues.

The second major difficulty stems from their location: all the *Slfn* paralogues are clustered in all species studied (3, 4) (Figure 2). In mapping studies, physical proximity, especially of related genes, constitutes a major problem for the unequivocal identification of the paralogue that is responsible for a given phenotype. *Slfn* clustering was probably the result of duplication events originated by unequal crossing over, although non-homologous mechanisms cannot be discarded.

Functional redundancy constitutes the third major challenge to the dissection of the biological role of each of the SLFN members. Although some family members have accumulated too many mutations as to code for functional proteins (3), it is unclear if, in other cases, one of the duplicated copies acquired new functions (neofunctionalization) or if the ancestral functions were partitioned among the duplicates (subfunctionalization), developing complementary functions (6). For instance, the first knock-out studied [that of mouse *Slfn1* (1)] showed no apparent phenotype, suggesting that the loss of this gene was compensated by the redundant functions of other *Slfn* products. Consequently, this knock-out provided no information about the function of SLFN1.

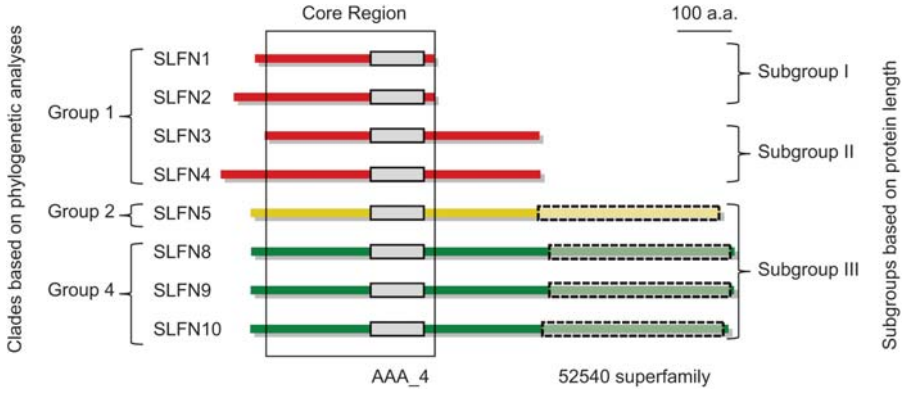

Figure 1 SLFN proteins in mouse.
They are grouped into four major clades (Groups 1–4) based on phylogenetic analyses [Figure 3; (3)] or three subgroups (I–III) based on their length (4). *Slfn14* (which is the only representative of Group 3) and *LOC435271* are predicted genes without sufficient expression support and, therefore, their products (and Group 3) have not been represented. Rectangles indicate the location of the common Core Region (3), the AAA_4 domain (Pfam04326) and 52540 superfamily (P-loop containing nucleoside triphosphate hydrolases superfamily) domain (www.ensembl.org). The latter domain is present in DNA and RNA helicases and some other proteins. Modified from (3) with permission.

Given the diversity, but also potential redundancy, of the *Slfn* genes, as well as their implications in their study, it is key to determine how many *Slfn* members exist, not only in mouse, but also in other species, and how they are distributed among species. Understanding the *Slfn* family origin and evolution can provide us with very valuable insights for the interpretation of the expression and functional data that has been accumulated over recent years. Moreover, the comparative analysis of these data from an evolutionary perspective will provide new clues for future studies of the *Slfn* gene family.

Number, characteristics and genomic location of the *Schlafen* family members in mouse

The study of the mouse *Slfn* genes has shown a number of interesting features: *Slfn1-10* genes size ranges from 992 to 23,806 nucleotides, and their protein products from 337 to 920 amino acids, with 31%–92% identity and 51%–96% of similarity over a common conserved domain of 312–331 amino acids (3) (Figure 1). According to their protein length, they have been classified into three subgroups: Subgroup I includes the shortest ones (SLFN1-2), Subgroup II includes SLFN3-4 and the longest, SLFN5-10, belong to Subgroup III (4, 7). Although it has been claimed that this classification is similar to the evolutionary and functional clustering of the family members, there are also substantial differences (see Figure 1 and sections below).

Additional homologous (paralogous) sequences found within the same genomic region overlap with *Slfn14* and *LOC435271* gene predictions. However, further data will be necessary to confirm the existence of functional protein products (2, 3, 5) (Figure 2). Interestingly, comparative analysis among genetically diverse mouse inbred strains has shown the presence of large insertions/deletions that include some *Slfn* members (5, 8), such as *Slfn8* (de la Casa-Esperon and Sapienza, unpublished data). Therefore, and specially in rodents (see *Slfn* evolution section below), the possibility of copy number variation among individuals/strains must be taken into account in many *Slfn* studies.

To date, there is very little knowledge about the isoforms expressed by the diverse *Slfn* genes (3), which surely will be revealed in future studies. The SLFN1-10 proteins share a common core region that contains a divergent AAA domain (AAA_4) with potential ATP binding activity (3, 4) (Figure 1). In their longer N-terminal region, SLFN5-10 also contain sequences like those present in proteins with nucleoside triphosphate hydrolase activity and similar to those found in AAA ATPases (3, 4). AAA ATPases (ATPases associated with diverse cellular activities) constitute a large family that includes proteins that participate in cell-cycle regulation, organelle biogenesis, protein proteolysis and disaggregation and intracellular transport, as well as molecular chaperones, helicases and transcription factors (9). Geserick et al. (4) suggested that several features of SLFN5-10 are shared by proteins with RNA helicase or RNA structure modeling functions. In general, ATPases have a non-ATPase N-terminal domain that participates in substrate recognition, followed by one or two AAA domains, one of which might degenerate (10) as it could be the case of several SLFN members (Figure 1). Although AAA ATPases can assemble to form oligomers (often hexamers) (11), it is still unknown if SLFN proteins have the ability to interact with each other.

In mouse, the *Slfn* genes are located within a 350 Kb region of chromosome 11 flanked by the *Unc45* and *Pex12* genes (Figure 2). In other mammalian species, they are also clustered within the same homologous (orthologous) region (Figure 2) (3). However, the number and relative location of the *Slfn* genes differs among them, suggesting that the evolution of this gene family has been shaped by multiple duplications (and maybe deletions), as well as lineage-specific inversions (3).

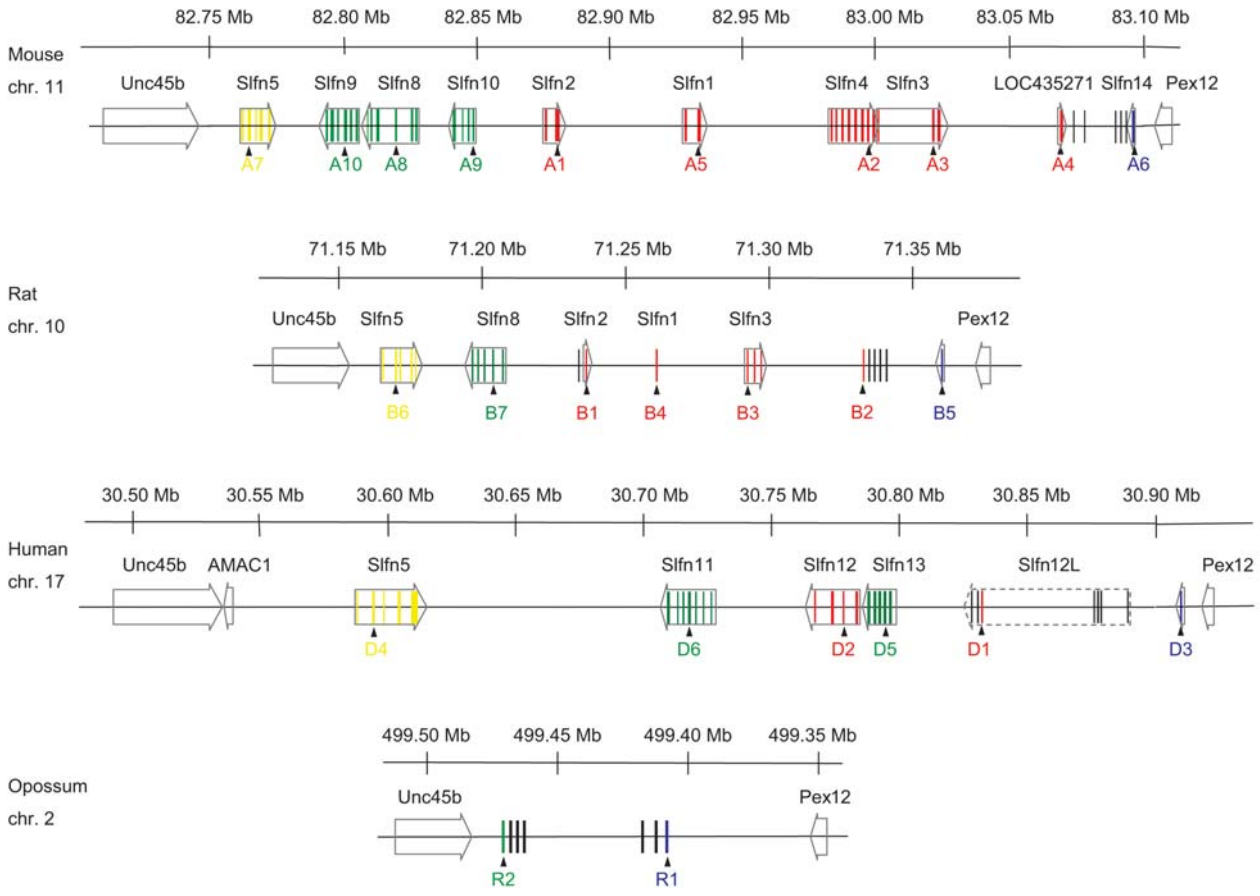

Figure 2 Orthologous regions in mouse, rat, human and opossum that contain the *Slfn* cluster.
Indicated are the Ensembl annotated *Slfn* genes and the *Slfn*-related sequences identified by Bustos et al. (3), their exons, and direction of transcription. The code and location of the sequences used in the phylogenetic analysis represented in Figure 3 is indicated below. Colors indicate their relationships according to the phylogenetic major clades (red=Group 1; yellow=Group 2; blue=Group 3; green=Group 4; black=unidentified). Opossum R2 sequence (which could not be included in Figure 3 phylogenetic analysis), was found to be more similar to *Slfn8, 9* and *10* (Group 4) sequences than to any other mouse sequences (3). Adapted from (3) with permission.

Origin, evolution and distribution among species of the *Schlafen* gene family

A recent search of *Slfn* homologues in the same and in different species (paralogues and orthologues, respectively) showed that members of this gene family are present in every mammalian genome with substantial coverage (except platypus, a monotreme) (3). Interestingly, they were also found in one amphibian (*Xenopus laevis*) and one fish species (*Callorhincuys milii*), but not in other non-mammalian species with extensive genome coverage (such as *Xenopus tropicalis* or *Danio rerio*). Moreover, similar sequences (*v-slfn*) were found in all sequenced genomes of orthopoxviruses (OPVs) (2, 3).

The widespread presence of *Slfn* genes in marsupial and placental mammals, but patchy presence in other orders of vertebrates, raised the question about the origin and evolution of this gene family. Neighbor-joining and Bayesian phylogenetic analyses of *Slfn* amino acid sequences of the conserved core region revealed four major clades [designated Groups 1–4, Figures 1 and 3] (3). Three are the major conclusions of these analyses: first, that genes of each of these major *Slfn* groups are present in most extant placental mammals and, therefore, the duplications that originated these groups occurred prior to the divergence between Laurasitheria and Supraprimates (Euarchontoglires) mammals (Bustos et al. 2009). Group 1 includes orthologues of mouse *Slfn1-4* and *LOC435271*. Group 2 is constituted by orthologues of *Slfn5* and Group 3 contains *Slfn14* orthologues. Moreover and in contrast with previous classifications based on protein length, the phylogenetic analyses indicates that *Slfn5* (Group 2) is more closely related to *Slfn1-4* (Group 1) than to *Slfn8-10* (3) (Figure 3). The latter are part of a separate clade designated as Group 4 and the inclusion in this group of the amphibian and fish sequences is arguable (3). In contrast with placental mammals, the analysis of the genome of opossum, a marsupial mammal, only revealed two *Slfn* sequences, belonging to Groups 3 and 4 (Figure 2).

The second conclusion of the phylogenetic analyses is that this gene family has more recently expanded several times in mammals (3). Duplications are particularly noticeable in Groups 1 and 4, in both rodents and primates. This has very

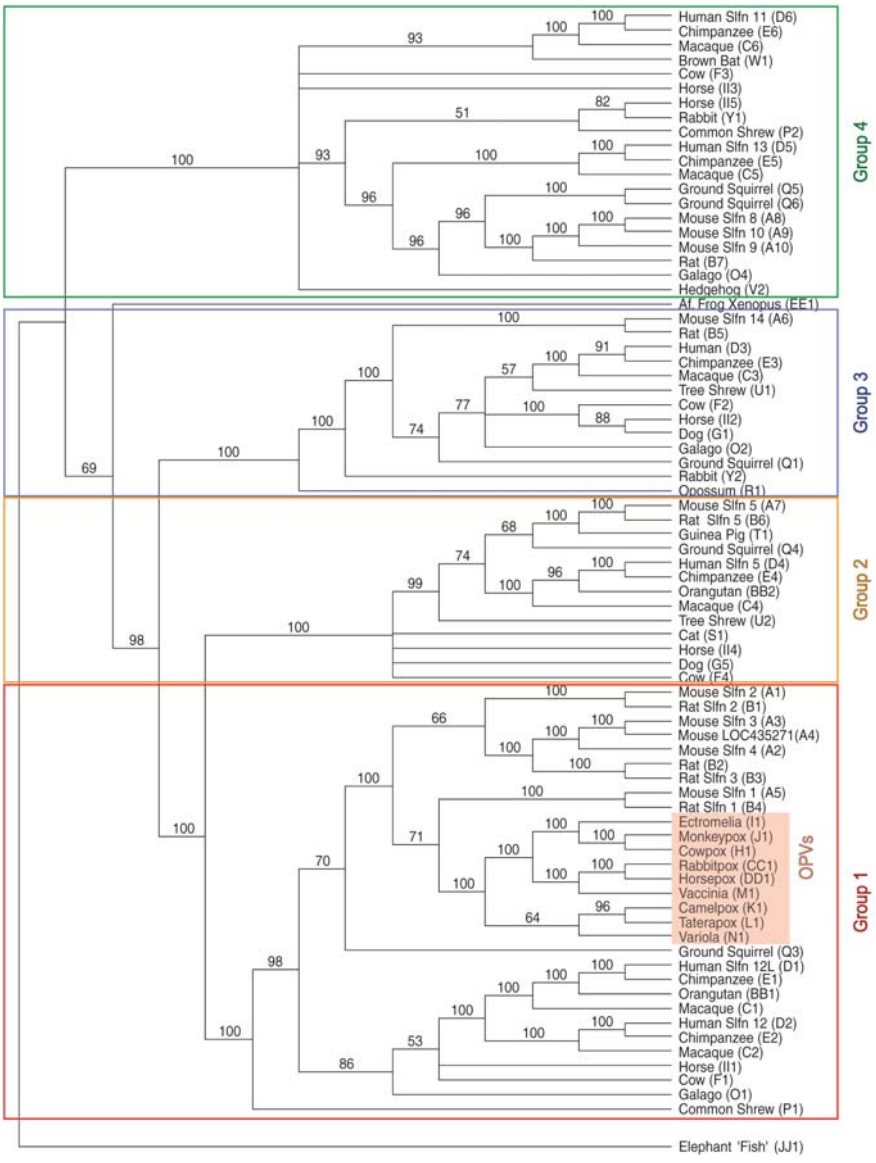

Figure 3 Phylogeny of *Slfn* genes based on Bayesian analysis of amino acid sequences orthologous to the mouse *Slfn* Core Region (see Figure 1).

The tree is rooted using the sequence from the chondrichthyan 'elephant fish' [in parentheses, sequence codes according to (3)]. Numbers at nodes represent posterior probabilities. Groups 1–4, as well as the orthopoxvirus sequences (OPVs), are indicated. Results from 4.5 million generations run under the 'mixed' (multiple model) criterion for evolution are shown. Modified from (3) with permission.

important implications in *Slfn* studies, especially for the extrapolation of the findings in one species to others. While mouse *Slfn1* and *Slfn2* have corresponding orthologues in the rat, mouse *Slfn3*, *Slfn4* and *LOC435271* are very closely related and originated from different lineage-specific duplications than those that resulted in rat *Slfn3* and an uncharacterized (B2) paralogue (Group 1, Figure 3). Moreover, in Group 1, primate *SLFN* genes also underwent an independent duplication, originating *SLFN12* and *SLFN12-LIKE* in humans. It is unclear which functions are conserved and shared by each one of the duplicates originated in the different species (12) and, therefore, comparisons between mouse *Slfn3* or *Slfn4*, rat *Slfn3* and human *SLFN12* functions should be taken with caution. In addition, it is unclear if their less characterized paralogues, human *SLFN12-LIKE* or mouse *LOC435271,* code for any functional product. Not all vertebrate and viral *Slfn* duplicates have retained open reading frames (ORFs) indicative of a functional role and, even in those with ORFs, there is little if any expression support for some of the *Slfn* copies (3).

A similar situation is found in Group 4 (Figures 2 and 3): while only one *Slfn* member has been identified in rat (*Slfn8*), at least two lineage-specific duplications produced mouse *Slfn8, Slfn9* and *Slfn10*. Although these three genes were recognized in the mouse C57BL/6 reference strain, copy number variation of these genes has been observed in other

inbred strains (de la Casa Esperon and Sapienza, unpublished data). An independent duplication event within Group 4 originated two copies in humans (*SLFN11* and *SLFN13*) and other species. Again, extrapolations between mouse SLFN8-10, rat SLFN8 and human SLFN11 and SLFN13 protein functions should be interpreted with special caution. Moreover, experimental artifacts such as probes, primers or antibodies cross-hybridization among very similar *Slfn* paralogues (e.g., mouse *Slfn8-10*) can lead to mixed results, unless the appropriate controls are taken.

The third conclusion that stems from the phylogenetic analyses, is that OPVs acquired *Slfn* sequences by horizontal transfer of a mammalian gene, most probably of rodent origin (3). As shown in Figure 3, the viral *Slfn* (*v-slfn*) genes are clustered in Group 1 and are closely related to mouse and rat *Slfn1-4*. *v-slfn* sequences have been found in all OPVs sequenced, located in orthologous positions, although not all conserve the ORF (1–3). Their close similarity and location in orthologous positions, as well as the accumulation of many substitutions along the ancestral branch of the viral sequences and the monophyletic origin of the OPVs (3, 13–15), suggest that the mammalian *Slfn* sequence was acquired only once by the progenitor of OPVs, although recombination among OPVs cannot be discarded (3).

In summary, the *Slfn* genes have expanded and diversified during the mammalian evolution, and are located in an unstable and rapidly changing region, specially in mouse and other rodents, where they could still be evolving fast (3, 5). Moreover, there is evidence of positive selection in the four groups, especially in groups 1 and 4. Most duplication events were followed by differential selection regime of the resulting paralogues, so that one of the sister *Slfn* genes evolved adaptively and diverged funtionally (3). Positive selection has often shaped the evolution of genes involved in the immune response (16, 17) and the *Slfn* genes do not appear to be an exception (see *Slfn* role in immune cells section).

In OPVs and once *Slfn* sequences were transferred from mammals, the *v-slfn* sequences may have acquired new roles or loss of some ancestral functions. This opens an interesting possibility: while mammalian SLFN products might participate in anti-viral immune responses, viral *v-slfn* could help to counteract them. Therefore, and in order to unmask the role of *v-slfn* copies in viruses, it is important to elucidate how the functions of the *Slfn* genes have evolved. Unfortunately and to date, comparative studies across species are limited, because mammalian studies have been restricted to three of them (typically mouse, but also human and rat). Nonetheless, recent studies have accumulated evidence for very important roles of the *Slfn* genes in diverse processes and have provided the first hints about their possible role in viruses and disease.

Expression and phenotypes associated to the mammalian *Schlafen* genes

Since the first studies of Schwarz et al. (1), changes in *Slfn* genes expression have been associated with cell growth and immune cells development phenotypes. The roles of these genes in the regulation of proliferation and differentiation have been broadly studied, although there is some controversy about some aspects. The two following sections will be dedicated to discussing these functions in more detail.

By mapping approaches, a number of phenotypes have also been associated with the *Slfn* gene family. Some may support their function in immune processes: for instance, several autoimmune disorders in human and mouse have been associated with genomic regions that contain *Slfn* genes (athough also other genes involved in immune processes, such as those of the *Ccl* gene family) (18–20). Genome-wide association studies have also related the human *SLFN* cluster with the levels of natural killer lymphocytes (21). Moreover, mapping studies in mouse have associated the *Slfn* region with two complete disparate phenotypes: DDK syndrome of embryonic lethality and meiotic drive. The DDK syndrome is observed in crosses between females of the DDK inbred strain and males of other inbred strains, producing embryos that die before implantation. This lethal phenotype is the result of the interaction of a factor provided by the sperm with a maternal cytoplasmic product present in the DDK oocytes (22–25). Both genes, the maternal and the paternal, map to the *Om* (*Ovum mutant*) region, which contains the *Slfn* genes; among them, *LOC435271* is the stronger candidate for the paternal incompatible allele responsible of the DDK syndrome (4, 5, 26–30). Interestingly, a second phenotype was observed in the *Om* region: unequal segregation of chromatids during the second meiotic division in mouse eggs (meiotic drive). As a result, particular *Om* alleles are transmitted to the offspring with more frequency than expected (28, 31–33). To date, and due in part to the difficulties posed by the *Slfn* family close location, similar sequence and putative redundancy, the role and the identity of the *Slfn* genes that contribute to these phenotypes are still unclear.

Expression analyses have shown that the *Slfn* genes are transcribed in reproductive organs, oocytes and early embryos, supporting a possible role of one/some of these genes in meiotic drive and embryonic lethality (3). Besides these cell types, *Slfn* genes are expressed in diverse mammalian tissues, with a clear preference for those related with the immune system, which agrees with a role of the *Slfn* genes in immune cells development and functioning (1, 3, 4, 34). In addition, changes in the RNA levels of diverse *Slfn* members have been observed during the development of cells of the immune system and in response to infections by several pathogens [and related molecules such as the lipopolysaccharide (LPS)], including viruses (1, 4, 34–42).

In contrast, minimal or undetectable expression of some/all *Slfn* members has been observed in other tissues or cell types analyzed, such as fibroblasts [although *Slfn* expression can be induced in the latter (35, 43)]. Nonetheless, many studies have analyzed the effect of the transfections of *Slfn* genes in fibroblasts, especially for the effect of these genes in cell growth regulation, sometimes with contradictory results (1, 4, 38, 41, 43, 44). A possible explanation for these contradictions is that the absent/residual expression of an endogenous *Slfn* could be accompanied with the absence of

the molecules that normally interact with the product of that gene, resulting in a number of functional artifacts when the same *Slfn* gene is artificially overexpressed. Further artifacts can be encountered when transfection of a *Slfn* gene of one species is performed into cells of other species. As in any cross-species studies, a *Slfn* transgene product might not be able to establish normal interactions with divergent molecules of a different species. The interpretation of cross-species studies is particularly complicated when lineage-specific duplications have originated additional *Slfn* copies in one or both species, with potentially divergent functions (such as Groups 1 and 4 *Slfn* genes, Figure 3) (7, 41, 45, 46).

At the cellular level, the location of several SLFN proteins has been characterized. Neumann et al. (7) found that mouse SLFN1, SLFN2 and SLFN4 were mostly cytoplasmic, while SLFN5, SLFN8 and SLFN9 were located in the nucleus. Although it should be noted that this study used 3′FLAG-tagged mouse *Slfn* genes transfected into human cells, some findings have been confirmed in other cell types. Preferential cytoplasmic location has been observed for mouse SLFN1 in thymocytes [although also nuclear (41)], at SLFN2 in mouse fibroblasts and macrophages (7, 43) and in rat T-cells for SLFN3 (34). In contrast, SLFN1 induced translocation to the nucleus has been reported in mouse fibroblasts and T-cells (41). Among the longer SLFN proteins, only one study supports SLFN5 nuclear location in human melanoma cells (47).

The *Schlafen* family roles in immune cells development and function

The *Slfn* family members participate in diverse aspects of the activation and differentiation of the cells of the immune system. These roles are supported by expression studies of *Slfn* genes in several immune cell types at different developmental stages and under diverse stimuli, as well as of transgenic and knock-out animals. Unfortunately, most expression analyses are restricted to one gene (or a subset of *Slfn* genes), to the detriment of a more global understanding of the expression regulation, distribution and function of this gene family. Consequently, the experimental designs employed to date have not allowed the uncovering of potential interactions and redundant functions among the diverse *Slfn* members, although they suggest that such might be the case.

Since the first *Slfn* studies, changes in the expression of these genes have been reported during lymphoid and myeloid development (1, 4, 41, 42, 44, 48). For instance, expression of *Slfn1* increases during thymocyte development, whereas that of *Slfn4* decreases (1, 4, 41). Upregulation of several *Slfn* genes expression has been observed during *in vitro* differentiation of monocytic leukemia M1 cells and ERMYB myeloid cells into macrophage-like cells (4, 7), while *Slfn4* is down-regulated during CSF-1-mediated differentiation of bone marrow progenitors into macrophages (42).

The activation of immune cells by diverse stimuli or infections is also accompanied by changes in the expression of several *Slfn* genes. Activation of peripheral T cells has been reported to result in increased levels of several *Slfn* members (such as mouse *Slfn3, Slfn4* and *Slfn9*, as well as rat *Slfn3*), while the expression of others decreases (e.g., mouse *Slfn1, Slfn2* and *Slfn8*) (1, 4, 34, 41). Whereas interferon-γ (IFN-γ) does not appear to affect these expression changes, it leads to upregulation of several *Slfn* genes in macrophages (4); in contrast, *Slfn4* has been reported to be a type I IFN target (42). LPS and CpG-DNA stimulations also induce the expression of several *Slfn* members in both mouse and human macrophages (4, 40, 42).

In addition, several inflammation and infection processes have been related to differential expression of *Slfn* genes. Rheumatoid arthritis (a macrophage-mediated disease) has been associated with a region that contains the *Slfn* cluster (19). Moreover, analysis of a mouse model of this pathology has shown increased levels of *Slfn1, Slfn2* and *Slfn4* in joints (42). Changes in *Slfn* genes expression have also been observed after *in vivo* and *in vitro* infections with *Brucella abortus*, *Listeria monocytogenes*, *Klebsiella pneumoniae* and reoviruses (4, 35–37). Interestingly, after low-dose infection with *K. pneumoniae*, the levels of *Slfn1, Slfn2* and *Slfn3* are higher in lung of the pneumonia-resistant C57BL/6 strain than in the susceptible C3H/HeJ mice (36). Whether *Slfn* copy number variations or other polymorphisms contribute to the infection susceptibility remains to be determined.

Transgenic and knock-out mice studies have shed some light over the role of individual *Slfn* genes in the immune system. Conversely, putative functional redundancy has obscured some results. Such is the case for mouse *Slfn1* and *Slfn3* knock-outs, for which no apparent phenotype in lymphocytes or monocytes was found (1, 49). In contrast, ENU-induced *elektra* mutation of *Slfn2* has provided new insights about the function of this gene. Homozygous mutant mice have an enhanced susceptibility to bacterial and viral infection and a diminished number of T cells and inflammatory monocytes. Interestingly, thymocytes seem to develop normally, while peripheral T cells and monocytes appear to acquire a semiactivated state that predisposes them to apoptotic death upon activation (49). The authors propose that, while normal naïve lymphocytes are sustained in a quiescent state waiting to respond to pathogen invasions, the *Slfn2* mutation disturbs the quiescence maintenance, resulting in apoptosis and immunodeficiency; therefore, they conclude that *Slfn2* contributes to the active maintenance of the T cells and monocytes quiescence (49, 50).

Slfn transgenic mice have been generated by restricting the expression of the transgenes to thymocyte lineages [those of *Slfn1* and *Slfn8* (1, 4)] or to myeloid cells [for *Slfn4* (42)]. Analysis of *Slfn1* transgenic mice showed a large reduction in thymus size and extensive loss of thymocytes (at several developmental stages) and T-cells (1). *Slfn8* overexpressing animals also displayed a substantial reduction in thymic cellularity, with impaired pre-T cell development and T-cell proliferation upon activation (4). The authors observed that endogenous *Slfn8* downregulation occurs at some stages and might be necessary for normal thymocyte development and T-cell activation, and suggested that the expression of the transgene interferes with these processes. Similarly, *Slfn4*

transgenic displays a reduction in the number of immune cells, in this case of monocytes and recruited inflammatory macrophages (42). The authors propose that downregulation of *Slfn4* expression is required during differentiation of the monocyte/macrophage lineage.

Therefore, analyses of *Slfn* transgenic mice support a role of the *Slfn* genes in the development and activation of cells of the immune system. However, they cannot provide information about the possible roles of this gene family in tissues other than thymic and myeloid lineages. Interestingly, knockout and transgenic animals can be excellent tools for exploring potential redundant/compensatory mechanisms among the *Slfn* members. For instance, studies of the effects of the absence or the overexpression of a given *Slfn* gene in the expression of other family members have not been performed to date, neither *in vivo* nor *in vitro*, but will surely contribute to unveil putative compensatory mechanisms (e.g., co-regulation or cross-regulation), as well as to identify redundant functions.

Schlafen genes participation in cell-cycle progression

As discussed in the previous section, the *Slfn* genes have been involved in proliferation and differentiation processes of cells of the immune system. In other cell types, several studies have also been focused on their contribution to cell cycle progression and growth. The first analyses of Schwarz et al. (1) reported that *Slfn1-3* transfections into mouse fibroblasts resulted in reduced colony formation; clones with induced *Slfn1* expression showed inhibition of cell-cycle progression, while clones of *Slfn2* and *Slfn3* could not even be established (1, 38). A similar observation was reported by Geserick et al. (4); in addition, these authors observed that the cell number reduction induced by *Slfn1* depended on the presence of the first 27 amino acids of this gene product, while no stable lines could be generated with the full-length. Further studies have reported that *Slfn1* induction correlates with a decrease in the levels of Cyclin D1 in fibroblasts, as well as in other cell types (38, 41). These studies concluded that *Slfn1* inhibition of cell-cycle progression occurs by preventing *Cyclin D1* transcription. This would require SLFN1 translocation from the cytoplasm to the nucleus, which is mediated by the DnaJB6 chaperone protein (41). However, Zhao et al. (44) have challenged these findings, because they did not observe any inhibitory effect of *Slfn1* (and *Slfn2*) overexpression in cell proliferation or *Cyclin D1* transcription, neither in fibroblasts nor in other cell lines.

Conversely, further studies with *Slfn2* have shown that siRNA-mediated down-regulation of *Slfn2* expression in mouse fibroblasts increases proliferation and *Cyclin D1* levels. Unlike *Slfn1, Slfn2* does not appear to be translocated to the nucleus, at least in mouse fibroblasts with induced interferon expression (43). IFNα, a cell growth inhibitor, stimulates the expression of *Slfn* genes (1, 2, 3, 5 and 8) in this cell type. siRNA-induced downregulation of *Slfn2* in mouse fibroblasts and bone-marrow derived hematopoietic progenitors reduces the antiproliferative response to IFNα, as well as increases anchorage-independent growth in soft agar (43). Aside from the controversial cell growth inhibitory role, *Slfn2* has been found to participate in RANKL-induced differentiation of a monocyte/macrophage cell line into osteoclasts. Lee et al. (51) observed that *Slfn2* expression is induced by RANKL during osteoclastogenesis, while siRNA-mediated downregulation of *Slfn2* inhibits this process.

Further studies of the Group1 *Slfn* members have been performed in rat *Slfn3* (which, despite the name, does not necessarily share common functions with mouse *Slfn3*, because they might have been split or retained by other co-orthologues generated by lineage-specific duplications since their common ancestor, see Figure 3). This gene is expressed in the colonic mucosa of aged rats at higher levels than in young ones (45). Administration of wortmannin, a colonic mucosal proliferation inhibitor, stimulated the expression of *Slfn3* in aged rats. Transfection of rat *Slfn3* into the HCT-116 human colon cancer cell line, also had an antiproliferative effect (45), in spite of the caveats of cross-species transfection studies already discussed; however, it is unclear if similar functions are played by any human *SLFN* gene product (particularly those of Group 1, see Figure 3). Nonetheless, two studies have reported the participation of rat *Slfn3* in the differentiation of the IEC-6 rat small intestinal cell line induced by diverse stimuli (46, 52).

Outside Group1 *Slfn* members, transfection of NIH-3T3 mouse fibroblasts with other mouse paralogues (*Slfn5, Slfn8, Slfn9* or *Slfn10*) did not reveal any antiproliferative effect (4). Conversely, studies in humans have shown that, like in mice (43), IFNα induced the expression of *SLFN* genes (*SLFN5, SLFN11, SLFN12* and *SLFN13*) in normal epidermal melanocytes (47). Although human *SLFN5* mRNA levels were lower in malignant melanoma cell lines than in a normal cell line, the expression of this gene was strongly upregulated upon IFNα induction. Moreover, down-regulation of *SLFN5* expression by siRNA in one melanoma line resulted in increased invasion and anchorage-independent growth and reduced the IFNα antitumoral effects (47). Further studies in humans and other species will be necessary to determine if the expression of the orthologous genes (mouse *Slfn5*, rat *Slfn5*, etc.) or of any other genes coding for long proteins (Group 2 and 4 *Slfn* members, Figure 1) have any antiproliferative effects.

Viral *Schlafen* sequences: a role in host immune response evasion?

Outside vertebrates, *Slfn* sequences have been identified in OPVs (1–3). These are viruses that infect mammals, including variola, the causative agent of smallpox, vaccinia (used in the vaccination that eradicated smallpox) and monkeypox, which has caused several recent outbreaks in humans (http://www.cdc.gov/ncidod/monkeypox/). Interestingly, *v-slfn* sequences are very similar and present in all sequenced OPVs (84%–99% similarity over 299–308 amino-acids), but are absent in any other viruses (3). There is only one *v-slfn*

copy per OPV genome, always in the same orthologous position. However, not all have retained an ORF: while monkeypox, cowpox, ectromelia (mousepox), taterapox and camelpox virus genomes code for putative functional proteins, the *v-slfn* sequences are fragmented in variola and vaccinia, as well as horsepox and rabbitpox viruses. In addition, the 5′ ends of the ORFs contain sequences related to the baculovirus p26 protein, of unknown function (1–3, 53).

Phylogenetic analyses have shown that *v-slfn* sequences are closely related to Group 1 mouse and rat *Slfn* genes (2, 3) (Figure 3). The analysis of *v-slfn* genes performed by Bustos et al. (3) indicates they were originated by horizontal transfer of *Slfn* sequences from a rodent to an OPV, where they subsequently diverged. Unlike mammalian *Slfn* genes, and in contrast with previous *v-slfn* studies (54), Bustos et al. (3) did not find indications of positive selection during the evolution of the OPV orthologues. On the contrary, their data suggested that the *v-slfn* sequences within ORFs evolved under purifying selection, which supports an important role of these genes in OPVs (3).

What is the role of the *v-slfn* genes in OPVs? The existence of intact ORFs in several of them suggests they have been retained because of some important function. Indeed, v-slfn proteins are detected in cells infected with OPVs that carry intact *Slfn* ORFs (such as monkeypox, cowpox and camelpox), but not in cells infected by viruses with fragmented *v-slfn* copies (e.g., vaccinia viruses) (2, 55). However, to date, there are only a few studies about the function of *v-slfn* genes. Early expression of these genes has been observed after camelpox and monkeypox infection (2, 55), which typically occurs in genes that participate in the regulation of intermediate gene expression, viral DNA synthesis, and modulation of the host anti-viral response. Transfection of mouse fibroblasts with camelpox *v-slfn* (with intact ORF) has no effect in proliferation; however, *v-slfn* lacks the 27 amino acids that appear to be necessary for mouse *Slfn1*-induced proliferation inhibition of fibroblasts (2, 4). In order to study the function of v-slfn during viral infection, Gubser et al. (2) generated vaccinia recombinant viruses in an attenuated strain. Vaccinia *v-slfn* does not code for a functional protein, so they introduced camelpox *v-slfn* intact ORF sequences, with and without HA-epitopes. *In vitro* infections of several cell types with these viruses (as well as with wild-type camelpox virus) showed that the v-slfn protein is present in the cytoplasm. However, v-slfn did not produce any apparent effect on recombinant vaccinia replication or plaque morphology (4). *In vivo* studies in a mouse model of intradermal infection also did not show any effect of v-slfn on the size of the lesion caused by vaccinia (2).

However, a murine model of intranasal infection showed conflicting results: on one hand and compared to control virus (without *v-slfn* ORF), infection with recombinant vaccinia viruses containing non-tagged camelpox *v-slfn* (or C-terminal HA-tagged) caused less weight loss and signs of illness; viral titers seemed to decay earlier than controls on lungs and were very low in spleen (2). The authors concluded that the expression of *v-slfn* did not prevent either viral infection or replication, although probably accelerated virus clearance by the immune system. An intriguing possibility suggested by Gubser et al. (2) is that *v-slfn* might actually decrease virus virulence, in order to avoid OPVs infections that would be too sudden and devastating for the host as to hinder viral spreading through the host population. But on the other hand, viral infection with N-terminal HA-tagged *v-slfn*-carrying recombinant vaccinia appeared to reduce body weight and mouse health as much as the control virus infection. Even more, viral titers in lungs and spleens were higher in the presence of this N-terminal tag than without it. This could be due to N-terminal modifications of *v-slfn*, because the HA-epitope seemed to be removed from the resulting protein (2). If such is the case, it would be interesting to determine the role of the N-terminal sequences of *v-slfn* in viral infection and replication.

Future studies will determine if the findings of Gubser et al. (2) are reproducible in other infection models, as well as by *v-slfn* of other OPVs. A recent study of the genome of a monkeypox strain that is resistant to cidofovir (an inhibitor of *in vitro* viral replication) with respect to a wild-type cidofovir-responsive strain, has identified, among several polymorphisms, one in the *v-slfn* gene (56). The polymorphic site of the cidofovir-resistant strain codes for a non-synonymous substitution in the *Slfn* sequences of the gene, although outside of the AAA-related domain. Whether this polymorphism affects viral replication remains to be determined.

The above studies have triggered a number of hypotheses about the role of the *vSlfn* in OPVs and their interplay with the host *Slfn genes*. Contribution of mammalian *Slfn* members to antiviral responses is supported by the observation of upregulation of mouse *Slfn2* and *Slfn3* expression in response to reovirus infections (35). The association of interferons with *Slfn* genes expression and effects (see previous sections) also suggests a participation of the products of these genes in the interferon-mediated anti-viral response. Moreover, Brady et al. (38) proposed that, since viruses often induce S-phase in order to enhance viral propagation, SLFN-mediated inhibition of Cyclin D1 induction of S-phase could prevent viral replication. Conversely, viruses could have used *Slfn* sequences in order to counterattack the host antiviral machinery. The acquisition of mammalian sequences by viruses is not a rare event: in fact, horizontal transfer of genes involved in the host immune response to viruses and selection of such genes to allow viruses to escape the host immune system has been observed in many instances (15, 57–59). Moreover, genes involved in virulence and modulation of the immune response are usually located in the terminal regions of OPVs, where *v-slfn* is found (60). Whether and how *v-slfn* genes contribute to OPVs ability to elude the host immune response is still unclear. One possibility could be through the formation of non-functional oligomers with the host SLFN proteins. Interaction with mammalian (or even viral) SLFN proteins might be feasible, because the AAA ATPases and the p26 baculovirus protein have been reported to form multimeric agregates (11, 53).

The study of the role of the *Slfn* genes products in host-viral interactions has very important implications. The proximity between viral and murine *Slfn* sequences is particularly

relevant, because it suggests that *v-slfn* proteins may play a special role in the OPVs infection of mice and other rodents. Smallpox could be eradicated because it only infects humans. However, other OPVs can infect and spread through diverse rodents. Such is the case of monkeypox, which can also infect humans and even be fatal (http://www.cdc.gov/ncidod/monkeypox/). Although the transmission efficiency through humans is low, any major outbreak that could result from changes in this virus would be very difficult to control, because rodents could act as natural reservoirs and disperse the disease (58). Monkeypox virus carries an intact *v-slfn* ORF that produces a protein upon infection (3, 55), which is mutated in a drug-resistant strain (56). Therefore, the study of *v-slfn* genes and their host orthologues could play an important role in the battle against OPV infections.

Expert opinion and outlook

Over the last years, the rapidly increasing number of *Slfn* studies and the relevance of their findings have brought a new interest towards a gene family that was barely known. Every function associated with the *Slfn* genes products has been shown to be of great importance: proliferation control and differentiation of diverse cell types, from embryos to aging cells, and immune response. Investigating the exact contribution of each of the *Slfn* members to these functions has an enormous interest, not only for the understanding of basic and key processes, but also because of their implications in disease and therapy. In this regard, *v-slfn* genes, which are unique to OPVs, have a special relevance in the design of therapies against OPV infections to humans. Because *v-slfn* genes originated from a mammalian copy that was horizontally transferred, and mammalian *Slfn* genes have been associated with the immune response, the interaction between host and viral *Slfn* genes constitutes a research avenue worthy of being explored.

The *Slfn* members were generated by multiple duplications and are clustered in a small and unstable region. Their proximity, similarity and putative redundancy have constituted major obstacles to dissect the functions of each of the family members. Consequently, characterization of the *Slfn* genes requires special attention in the identity of the member under study, in order to avoid mixed results with other family members. The diversification of several *Slfn* clades, especially in rodents and primates, is also an important matter to be taken into consideration when working across species.

This diversification of the *Slfn* members also opens very interesting avenues for their research. Have new functions been acquired or ancestral functions lost after lineage-specific duplications? Functional diversification has very important implications in comparative studies across species. And even within species, what are the consequences for the new duplicates? A key question to be answered is if paralogues have redundant functions. Future studies will be aimed to elucidate the interplay among the diverse *Slfn* members within a species – for example, if changes in the expression of one gene affect the others. It is also unknown if SLFN proteins can form homo- or heteroligomeres and the consequences of potential interactions with different SLFN proteins. Functional redundancy can be also investigated by analyzing the consequences of the loss of a *Slfn* member – i.e., how *Slfn* genes contribute to compensate the loss of one of them. Knock-out studies (1, 49) and copy number variation [which has been observed in mice (de la Casa Esperon and Sapienza, unpublished results) and could occur in other species] have demonstrated that some *Slfn* members are dispensable. Moreover, *Slfn* copy number variation is an important factor to take into account when working with genetically diverse individuals or with mouse inbred strains other than C57BL/6, due to the sequence similarity and probable redundancy of additional duplicates. Also, because it opens a new field of study of the potential implications of *Slfn* copy number variation in diverse phenotypes and in the susceptibility to infections.

Highlights

The *Schlafen* gene family was discovered in cells of the immune system and has been associated with the control of proliferation and differentiation processes in these, as well as in other cell types. Other phenotypes related to the *Schlafen* cluster are immune responses, embryonic lethality and meiotic drive.

Schlafen members are present in mammals in variable number, as well as in orthopoxviruses, which acquired one copy by horizontal transfer of sequences of mammalian (probably rodent) origin.

The study of the *Schlafen* gene family has very important implications due to the critical functions they have been associated with, especially in infections and disease. Future studies will be focused on the characterization of the specific role of each *Schlafen* gene product in diverse tissues and species, as well as in the potential interplay of the diverse *Schlafen* members that may result in redundant (and even opposite, as in the case of virus and hosts products) functions.

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