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Caveolins in rhabdomyosarcoma

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Abstract

Caveolins are scaffolding proteins that play a pivotal role in numerous processes, including *caveolae* biogenesis, vesicular transport, cholesterol homeostasis and regulation of signal transduction. There are three different isoforms (Cav-1, -2 and -3) that form homo- and hetero-aggregates at the plasma membrane and modulate the activity of a number of intracellular binding proteins. Cav-1 and Cav-3, in particular, are respectively expressed in the reserve elements (*e.g.* satellite cells) and in mature myofibres of skeletal muscle and their expression interplay characterizes the switch from muscle precursors to differentiated elements. Recent findings have shown that caveolins are also expressed in rhabdomyosarcoma, a group of heterogeneous childhood soft-tissue sarcomas in which the cancer cells seem to derive from progenitors that resemble myogenic cells. In this review, we will focus on the role of caveolins in rhabdomyosarcomas and on their potential use as markers of the degree of differentiation in these paediatric tumours. Given that the function of Cav-1 as tumour conditional gene in cancer has been well-established, we will also discuss the relationship between Cav-1 and the progression of rhabdomyosarcoma.

Keywords: rhabdomyosarcoma • caveolins • skeletal muscle

Introduction

Caveolins (Cav-1, -2 and -3) are membrane scaffolding proteins that regulate numerous processes in a variety of tissues and cell types [1, 2]. They participate in the biogenesis of *caveolae*, flask-shaped invaginations of the plasma membrane where several molecules involved in the regulation of transduction pathways are specifically and highly enriched [3–5]. Caveolins are anchored at the inner leaflet of *caveolae* through a short hairpin hydrophobic domain and protrude towards the cytoplasm, where they can bind and influence the activity of several protein partners *via* a caveolin scaffolding domain (CSD) [6, 7]. In skeletal muscle, in particular, Cav-1 expression is restricted to satellite cells [8–10], whereas Cav-3 plays a pivotal role in the mature myofibres [11–13], as demonstrated by the fact that its deficiency is associated to a set of genetic muscular disorders, known as caveolinopathies [14–17]. Rhabdomyosarcoma (RMS) is the most frequent soft tissue sarcoma occurring in childhood and sharing features of myogenic cells [18–20]. Hence, cancerous RMS cells can be univocally identified by means of muscle markers [21]. Two different

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works have shown that caveolins are expressed in a cell stage–dependent manner in RMS [22, 23]. This review will summarize these findings and highlight the relevance of caveolins as diagnostic markers for the detection of RMS with a different grading. In addition, the potential contribution of caveolins to the progression of RMS is discussed, particularly for Cav-1.

Caveolae and caveolins

Caveolae are characteristic flask-shaped invaginations of the plasma membrane that configure as specialized microdomains involved in numerous functions, including cell signalling, lipid regulation and endocytosis [1–7]. Cav-1, Cav-2 and Cav-3 are the main structural proteins of *caveolae* and are codified by three different genes that have a high degree of homology [1, 2] (Table 1). In particular, caveolins are anchored to the inner leaflet of the plasma membrane *via* a short hydrophobic loop, which allow them to assume a unique hairpin structure, characterized by the presence of both N- and C-terminal portions facing towards the cytoplasm [1, 2]. Cav-1 and Cav-2 form hetero-oligomers and are ubiquitously co-expressed [24], whereas Cav-3 forms homo-oligomers that are predominantly expressed in skeletal and cardiac muscle [11, 12] (Table 1). Cav-1 and Cav-3 are unequivocally required for the biogenesis of *caveolae* [25], as corroborated by the lack of *caveolae* in Cav-1 null animals [26] and in muscle and cardiac tissues of Cav-3 null animals [27, 28], despite other key protein molecules, such as PTRF-Cavin [29, 30] and ARAF-1 [31], seem to have a role in the formation of these membranous structures. Although caveolins are predominantly recovered at the plasma membrane and in Golgi apparatus of cells [32–34], their subcellular localization may change upon post-translational modifications, as occurs for Cav-1, which may be targeted to cytoplasm or secretory vesicles upon phosphorylation on Tyr14 or Ser80 residues, respectively [35,36].

Caveolins as scaffolding proteins

The most important feature of caveolins relies on their ability to bind and modulate the biological activity of a multitude of intracellular protein partners through the so-called CSD, which recognises motifs enriched of aromatic residues [6, 37]. Although the CSD sequence results highly conserved in both Cav-1 and Cav-3, it is slightly different for Cav-2, which in fact seems to be clearly less prone to bind proteins. To date, more than 90 proteins are known to be bound and regulated by caveolins, including G-protein–coupled receptors and G-proteins, different membrane receptorial (insulin receptor, PDGFR, TGF-β receptors, *etc.*) and non-receptorial proteins (Src, Fyn, PKA, *etc.*), enzymes (adenylyl cyclase, e- and nNOS, phospholipases, *etc.*), GTPases (H-RAS, RhoA, *etc.*), protein adaptors (Shc), nuclear proteins (estrogen and androgen receptors) as well as a miscellaneous of other proteins involved in disparate processes (E-cadherin, β- and γ-catenin, calsequestrin, calreticulin, *etc.*) [1–7]. Cav-1, furthermore, is a cholesterol-binding protein and contributes to regulate its homeostasis [38].

Table 1 The family of caveolins: genomic localization, cell- and tissue-specific expression and principal knockout mouse phenotypes

Human gene	Chromosomal localization	Expression patterns	Knockout mouse phenotypes
Cav-1	7q31.1	Adipocytes	Diabetes [40, 41]
		Cardiac fibroblasts	Lung diseases [26, 42, 43]
		Endothelia	Heart diseases [42, 44]
		Macrophages	Cerebral ischaemia [45–47]
		Neural cells	Predisposition to skin and breast cancer [54, 75–79]
		Pneumocytes	Protection from prostate cancer [80]
		Smooth muscle cells	
		Striated muscle cells	
Cav-2	7q31.1	Same as *Cav-1*	Impaired pulmonary functionality [48]
			Abnormalities in skeletal muscle [49]
Cav-3	3p25	Striated muscle cells	Mild myopathic changes [27, 28]
		Smooth muscle cells	Cardiomyopathy [50]
		Cardiac myocytes	Insulin resistance and increased adiposity [51]

Impact of the lack of caveolins in the whole body physiology

Mice deficient in Cav-1, Cav-2 or Cav-3 are viable and fertile but display several alterations in the whole body physiology [15, 39] (Table 1). In particular, Cav-1 null mice develop a complex spectrum of diseases, such as diabetes [40, 41], impaired lung [26, 42, 43] and heart [42, 44] functionality and cerebral ischaemia [45–47]. Moreover, Cav-1 null mice are predisposed to certain tumours whereas are protected from others [15, 39] (for a more detailed discussion please refer to section 'Relevance of Cav-1 as tumour conditional gene'). Cav-2 null mice show exercise intolerance associated to impaired pulmonary functionality [48] and peculiar abnormalities in skeletal muscle, such as tubular aggregate formation, mitochondrial proliferation/aggregation and increased number of satellite cells [49]. On the other hand, because of the restricted

tissue-specificity of Cav-3 expression within mature myofibres and cardiac myocytes, Cav-3 null mice exhibit mild myopathic changes, such as mononuclear cell infiltration, variable fibre size and presence of necrosis [27, 28], as well as a progressive cardiomyopathy due to extracellular regulated kinases (ERK) pathway hyperactivation [50]. In addition, Cav-3 null mice develop insulin resistance and increased adiposity [51], suggesting an involvement of Cav-3 in the regulation of the whole body glucose metabolism.

Relevance of *Cav-1* as tumour conditional gene

The human *Cav-1* gene is localized on a suspected tumour suppressor locus of chromosome 7q31.1 [52]. Targeted down-regulation of Cav-1, indeed, promotes cell transformation of NIH3T3 fibroblasts, anchorage-independent growth *in vitro* [53] and tumour growth *in vivo* [54, 55]. In addition, Cav-1 overexpression blocks mouse embryonic fibroblasts in the G_0/G_1 phase of the cell cycle [56] and abrogates the transformed cell phenotype [57, 58], suggesting that Cav-1 acts as a tumour-suppressor in non-neoplastic tissues by mainly limiting the ERK signalling pathway [53, 59]. Accordingly, Cav-1 is down-regulated in different tumours, such as ovarian [60], lung [61] and breast carcinomas [57, 62–64], mesenchymal sarcomas [65] as well as in cell lines derived from human tumours or transformed by oncogenes [66]. Paradoxically, Cav-1 is up-regulated in many other malignancies, such as colon adenocarcinoma [67], bladder carcinoma [68], oesophageal squamous cell carcinoma [69] and prostate cancer [70], suggesting that Cav-1 plays a dual role in cancer progression depending on the different type and stage of cancer [71–74]. The complex relationship between Cav-1 and cancer has been particularly outlined by the employment of Cav-1 null mice (Table 1). In particular, although ablation of Cav-1 seems to be not sufficient to induce spontaneous tumour formation, it significantly predisposes mice to skin and breast tumours by stimulating cellular hyperplasia [54, 75–79]. In striking contrast, genetic loss of Cav-1 in the TRAMP model (transgenic adenocarcinoma of mouse prostate) decreases incidence of prostate tumours and metastasis [80]. Collectively, a growing body of evidence derived from different models suggests that loss of Cav-1 cooperates to cell transformation in the early phases of tumour growth, whereas a later Cav-1 re-expression favours tumour metastases and multi-drug resistance [81–84]. A possible explanation to this ambiguous behaviour could be referred to the presence of multiple alterations in the cellular environment, such as the expression of different subsets of caveolin partners during tumour progression or the occurrence of inactivating mutations [85, 86] or post-translational modifications of Cav-1 [87, 88], which may promote a shift of Cav-1 activity from tumour-suppressor to proto-oncogene. In this sense, Cav-1 should be considered a tumour-conditional gene [71–74].

Role of caveolins in skeletal muscle

In skeletal muscle, Cav-1 and Cav-3 reside in satellite cells [8–10] and myofibres [11–13], respectively. Interposed among the fibres, satellite cells represent a pool of reserve elements which are recruited in different processes, such as the repair of damaged fibres [89], stretch [90] and fibre hypertrophy [91]. In particular, Cav-1 has been shown to play an important role in maintaining the quiescence of satellite cells by antagonizing the RAS/ERK signalling [10]. On muscle injury, Cav-1 is transcriptionally down-regulated through an HGF/cMET axis pathway, allowing the satellite cells to escape quiescence, migrate and repair the injured site [10]. On the other side, in mature myofibres Cav-3 regulates the activity of different signalling proteins [14–16], associates with the T-tubules structures [27] and stabilizes the dystrophin–glycoprotein complex through a WW-like binding domain [92, 93]. In this regard, a deficit of Cav-3 expression due to inherited Cav-3 gene mutations is responsible of a set of distinct neuromuscular and cardiac disorders [14–17]. For instance, the missense Cav-3 (P104L) substitution, which has been predominantly associated to the Limb Girdle Muscular Dystrophy 1-C (LGMD1-C) [94], affects the integrity of skeletal muscle *in vivo* [95, 96] and the myogenic program *in vitro* [97]. Remarkably, the homeostasis of skeletal muscle is even compromised by an excess of Cav-3, as observed in muscles derived from patients affected by Duchenne's muscular dystrophy [98, 99] and confirmed by studies of Cav-3 overexpression [97, 100]. Collectively, these observations suggest that the switch from Cav-1 to Cav-3 expression represents a pivotal step for the overall myogenic program, leading the muscle precursors from quiescence towards differentiation.

Histopathological, genetical and molecular signatures of RMS

Soft tissue sarcomas arise from primitive mesenchymal cells located throughout the body and make up to approximately 7% of all cancer cases in patients under the age of 20 [101, 102]. These tumours can be subdivided into two major groups: RMS and non-RMS soft tissue sarcomas, the latter including a miscellaneous of tumours, such as the synovial sarcoma, malignant fibrous histiocytoma, malignant peripheral nerve sheath tumour and fibrosarcoma [101]. The immunohistochemical or molecular detection of myogenic regulatory factors, such as MyoD and myogenin [103–106], allows an RMS diagnosis, whereas the detection of myosin and other contractile proteins identifies more mature RMS phenotypes [107]. The current classification of RMS into two major histological variants, termed embryonal (ERMS) and alveolar (ARMS), is supported by histopathological criteria and genetic signatures (Fig. 1).

Embryonal RMS

ERMS accounts for up to 80% of RMS in children of less than 10 years of age and is the most common and more treatable subtype. ERMS can occur at any site, including nasopharynx and biliary tract, but they are most commonly observed in the head and

Principal chromosomal rearrangements	**ERMS -** LOH and LOI on 11p15.5 [111-113] and LOH on 9q22 loci [120,121]		
	ARMS - Translocations t(2;13)(q35;q14) and t(1;13)(p36;q14) [126-128]		
Principal alterations in molecular pathways	p53 [141-145]	VEGF [154-156]	
	HGF [146,147]	FGFR4 [157]	
	IGF1-R [148-150]	IL-4R [158,159]	
	EGFR/HER [151,152]	RAS [122-124]	
	PDGFR and ligands [150,153]		
Principal alterations in molecular targets	PAX3/7-FKHR [126-128]	CDKs [166]	
	cMET [160]	H19 [114]	
	IGF-2 [110,117]	CDKN1C (p57/KIP2) [115]	
	IGFBP5 [161]	SLC22AIL (BWR1A) [116]	
	N- and C-MYC [162-164]	PTCH [120,121]	
	MDM2 [144,165]	MyoD [167,168,170-172]	
RMS mouse models	$p53^{+/-}$ and $^{-/-}$ [173,174]		
	$p53^{-/-}$ and FOS $^{-/-}$ [175]		
	$p53^{+/-}$ and activated HER-2/neu [176]		
	$p53^{+/-}$ or $^{-/-}$ and K-RAS [177]		
	PAX3-FHKR conditional transgenic [178]		
	PTCH $^{+/-}$ [179]		
	HGF transgenic [146] and with inactivated INK4a/ARF locus [147]		
	Dystrophin-deficient mdx mice [180]		
RMS zebrafish models	Activated K-RAS [181]		
			MIM ID
Human syndromes associated to RMS	Li-Fraumeni syndrome (germline p53 mutations) [141]		151623
	Beckwith-Wiedemann syndrome (mutations in 11p15.5 locus) [119]		130650
	Neurofibromatosis-1 (mutation in NF1) [182]		162200
	Costello syndrome (mutation in H-RAS) [183]		218040
	Gorlin syndrome (mutation in PTCH1 or PTCH2) [184]		109400
	Retinoblastoma (mutation in RB1) [185]		180200
	Mosaic variegated aneuploidy syndrome (mutation in BUB1B) [186]		257300
	Mismatch repair deficiency syndrome [187]		276300

Fig. 1 Molecular alterations, animal models and human syndromes associated to RMS. MIM ID numbers linked to each syndrome can be used to retrieve further informations at the following site: http://www.ncbi.nlm.nih.gov/omim.

neck or genitourinary region [108]. On histologic examination, ERMS are highly heterogeneous, ranging from poorly differentiated lesions with immature tumour cells to highly differentiated lesions containing rhabdomyoblasts with large eosinophilic cytoplasm. ERMS also comprise different histological subtypes formed by botryoid and spindle cells. A severe genomic instability generally characterizes ERMS subsets (Fig. 1): loss of heterozygosis (LOH) and the loss of imprinting (LOI) on chromosome region 11p15.5 are the most frequent signatures that retrieve the inactive allele and cause the loss of the active one [109–113]. This genetical signature

impairs the expression of different putative tumour suppressor genes on chromosome 11, including *H19* [114], *CDKN1C* (*p57/KIP2*) [115] and *SLC22AIL* (*BWR1A*) [116]; in contrast, the gene encoding for *IGF-2*, imprinted in the opposite direction, is overexpressed [110,117]. In accordance, the tumourigenesis of the RMS RD cell line is suppressed by transferring a normal human chromosome 11 [118]. Interestingly, inherited alterations of the 11p15.5 locus are retrieved in patients affected by the Beckwith–Wiedemann syndrome, an overgrowth syndrome associated to an increased risk of developing Wilms tumour, hepatoblastoma, adrenocortical carcinoma, neuroblastoma and also RMS [119]. LOH is also frequently observed on chromosome 9q22 (Fig. 1), causing deficiency in *Patched* (*PTCH*) gene [120] and predisposing to high incidence of medulloblastoma and ERMS [121]. In addition, even activating mutations in RAS gene, which is intriguingly localized within the 11p15.5 locus, are associated to ERMS [122–124].

Alveolar RMS

ARMS mainly affect adolescents and adults and are characterized by a poorer prognosis. ARMS cells resemble lung alveoli, with clusters of eosinophilic tumour cells arranged loosely and disposed in an alveolar pattern. ARMS typically occur in the trunk and body extremities [108] and frequently harbour non-random chromosomal translocations [125] (Fig. 1). In particular, translocations t(2;13)(q35;q14) and t(1;13)(p36;q14) account respectively for about 70% and 10% of ARMS and give rise to chimeric proteins that are formed by the fusion of the paired and homeo-DNA binding domain of PAX3 or PAX7 factors with the transactivation domain of FKHR (FOXO1) [126–128]. The so-called PAX3-FKHR and PAX7-FKHR transcription factors enable an aberrant transcriptional program and significantly contribute to RMS progression through multiple mechanisms. In particular, PAX3-FKHR and PAX7-FKHR are frequently overexpressed in ARMS [129], display incremented accessibility to chromatin due to localization exclusively nuclear [130–132] and reach a 10- to 100-fold increase in the transcriptional activation of downstream target genes in comparison to wild-type PAX3 and PAX7 proteins [133, 134]. Ectopic expression of PAX3-FKHR triggers a transformed phenotype in chicken embryo [134] and NIH3T3 fibroblasts [135] and increases the tumourigenicity of two ERMS cell lines [136]. Moreover, PAX3-FKHR prevents apoptosis [137, 138] and even abrogates myoblast terminal differentiation [139, 140]. Transgenic mice carrying PAX3-FKHR develop defects in hindlimb skeletal muscle formation and neural crest migration [140], but do not undergo tumour formation, supporting the idea that PAX3-FKHR expression is required but is *per se* not sufficient to cause oncogenic transformation.

Principal pathways and targets deregulated in RMS

The network of pathways deregulated in RMS is rather complex (Fig. 1). The loss of p53 seems to be central for RMS development [141–145], in addition to the deregulation of components for receptorial signalling pathways, including HGF [146, 147], IGF1-R [148–150], EGFR/HER-1, HER-2 and HER-3 [151,152], PDGFR [150, 153], VEGF [154–156], FGFR4 [157] and IL-4R [158, 159]. The overactivation of the RAS pathway occurs rather frequently [122–124]. In addition to the peculiar PAX3/7-FKHR expression [126–128], there are other molecular signatures that are commonly retrieved in RMS, such as the overexpression of cMET/HGF receptor [160], IGF-2 [110, 117], IGF-2–binding protein (IGFBP5) [161], N- and C-MYC [162–164], MDM2 [144,165] and some mutations in the *cyclin-dependent kinases* (*CDKs*) genes [166]. Moreover, as previously mentioned (section 'Embryonal RMS'), loss of expression of *H19* [114], *CDKN1C* [115], *SLC22AIL* [116] and *PTCH* [120,121] may occur. It is worth remembering that RMS cells are committed to myogenic lineage and therefore express muscle markers. In this regard, a paradoxical feature of RMS cells is that the expression of MyoD does not overlap with its full functionality because of multiple altered mechanisms [167], such as the inactivation of the mitogen-activated protein kinase (MAPK) p38 [168], whose function is required to enable MyoD transcriptional activity [169], or the presence of E-proteins complexes that compete for the generation of active full-length E-protein/MyoD heterodimers [170]. Moreover, PAX3-FKHR factor has been shown to increment the transcriptional levels of MyoD, but then abrogates its activity through protein phosphorylation [171, 172].

Animal models and human syndromes associated to RMS

A growing body of evidence derived from different animal models suggests that the development of RMS frequently occurs upon suppression of p53 pathway in conjunction with deregulated activities of receptorial systems along the RAS axis and/or the expression of *PAX3/7-FKHR* gene products (Fig. 1). In particular, RMS development occurs in p53-mutant mice [173, 174] and tumour incidence increases in conjunction with loss of FOS [175], activation of HER-2/neu [176], RAS [177] or the expression of PAX3/7-FKHR proteins [178]. In addition, mice with ablated *PTCH* gene [179] or with aberrant HGF signalling [146, 147] and non-transgenic dystrophin-deficient mdx mice [180] are prone to develop RMS. In zebrafish, transgenic expression of oncogenic K-RAS in activated satellite cells predisposes to RMS and simultaneous p53 inactivation accelerates tumour formation [181]. Finally, important clues on the risk factors predisposing to RMS came also from the evidence of a significant RMS incidence in certain familial cancer syndromes [145], including the Li–Fraumeni syndrome [141], Beckwith–Wiedemann syndrome [119], neurofibromatosis-1 [182], Costello syndrome [183], Gorlin syndrome [184], retinoblastoma [185], mosaic variegated aneuploidy syndrome [186] and mismatch repair deficiency syndrome [187] (Fig. 1).

Origins of RMS

Despite great efforts have been made to understand the molecular signatures of RMS, the identity of the tumour-initiating cell

remains to be clarified. Satellite cells and multipotent mesenchymal stem cells (MSCs) are thought to be the most credited source for ERMS and ARMS, respectively [188–190]. In particular, inactivation of p53 cooperates with oncogenic RAS in muscle precursors and satellite cells to induce ERMS in mice [191] and zebrafish models [181]. On the other side, despite the delivery of PAX3-FKHR or PAX7-FKHR in MSCs derived from mouse bone marrow is effective to induce expression of MyoD and myogenin, ARMS tumours form only in conjunction with the expression of a dominant-negative p53, SV40 early region or a constitutively activated H-RAS [192, 193]. These findings strongly support the notion that PAX3/7-FKHR factors may confer the myogenic identity to MSCs elements [188], but then secondary deregulated mechanisms are required to induce tumour formation.

Expression of caveolins in RMS tumours and cell lines

Validation of caveolins expression in RMS has drawn the attention only recently. In a large screening of different tumours, including RMS, malignant mullerian tumour and a spectrum of different neoplasms, Cav-3 was indicated as specific marker for the detection of mature RMS, being particularly expressed in those cell elements with abundant eosinophilic cytoplasm and striation [22]. In a recent study, we have confirmed this evidence by means of immunohistochemical analyses [23]. In particular, Cav-3 and Cav-1 were predominantly associated to mature or immature RMS tumours, respectively (Fig. 2). Given the heterogeneity in degree of maturation present in the RMS cell components, the expression of Cav-1 or Cav-3 cannot be univocally associated to a certain RMS histotype. Nevertheless, a simplified model would indicate such a relationship between the expression of Cav-1 or Cav-3 and a status of poor or advanced cell differentiation, respectively, as analogously observed in skeletal muscle (Fig. 2) [10]. To further substantiate the *in vivo* findings, the expression of caveolins has been analysed *in vitro* [23]. In particular, Cav-1 expression was retrieved in the majority of the human ERMS cell lines analysed (Fig. 3A), except for TE671 cells (personal unpublished results), which intriguingly displayed low expression of MyoD. This evidence should deserve attention as might suggest an unappreciated relationship between MyoD levels and the expression of Cav-1 in immature muscle precursors, as much as a relationship between myogenin and the levels of Cav-3 has been already shown in differentiating myoblasts [194, 195]. The analysis of Cav-1 behaviour was then particularly characterized by employing RD cells, an RMS model line in which *RAS* mutations are long known to counteract the myogenic differentiation *via* hyperactivation of the ERK pathway [196]. In RD cells, Cav-1 was found to be properly localized at the plasma membrane (Fig. 3B), thus excluding the presence of inactivating gene mutations leading to protein mislocalization, as occurs in some cancer types [62, 85, 86]. In particular, high levels of Cav-1 were associated to proliferation of

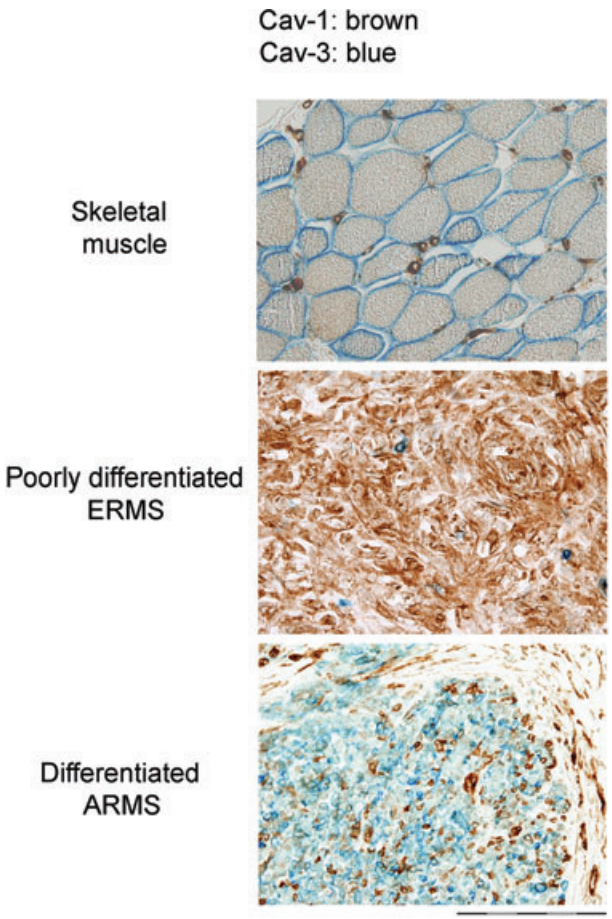

Fig. 2 Expression of caveolins in RMS tumours. Double immunostain showing that in skeletal muscle Cav-1 and Cav-3 mark satellite cells and the plasmalemma of myofibres, respectively. In RMS, Cav-1 and Cav-3 are predominantly associated to immature and mature tumours, respectively. Bars = 50 μm.

RD cells, whereas pharmacological inhibition of the ERK pathway, eliciting block of cell growth and subsequent differentiation, lead to Cav-1 down-regulation and increase of Cav-3, myogenin and MHC (Fig. 3C). Although it remains to be established if the decrease in Cav-1 might be related to growth arrest alone or to both withdrawal from cell cycle and subsequent myogenic differentiation, Cav-1 and Cav-3 seem to be associated to an immature and mature RMS cell phenotype, respectively, thereby confirming the previous *in vivo* observations. Keeping in mind that activating *RAS* mutations are frequently detectable in ERMS cells lines [122–124] and are critically involved in RMS tumour formation [177, 181, 183, 190–193], the relationship between Cav-1 expression and the RAS/ERK pathway deserves current attention. In fact, although Cav-1 is a marker of quiescence in muscle satellite cells [10], it configures as a marker of proliferation in RMS cells, suggesting that deregulated mechanisms in RMS cells might impair the ability of Cav-1 to overcome the RAS/ERK pathway. In this

perspective, it is crucial to assess how targeted silencing of Cav-1 might influence RMS cell growth. Importantly, expression of Cav-2 has been also observed in RMS (personal unpublished data). Cav-2, indeed, is almost always co-expressed with Cav-1 and its phosphorylation can regulate the formation of *caveolae* and mitosis [197, 198]. Thus, the presumed impaired ability of Cav-1 to control cell proliferation in RMS cells needs to be ascertained also in relation to Cav-2 functionality.

Future perspectives: assessing the role of caveolins in RMS tumour progression

At the plasma membrane caveolins control the activity of several proteins involved in pathways transduction, thereby representing a checkpoint of cellular signalling. As such, in the following paragraphs the potential relationship between caveolins and different pathways which are central to RMS development will be discussed (Table 2).

Cav-1 and p53 signalling

Inactivation of p53 pathway, as occurs for spontaneous p53 germline mutations [141] or overexpression of MDM2 [144, 165], plays a critical role for RMS development. As effect of p53 loss of function, cancerous cells elude senescence and divide indefinitely. In this context, cellular senescence may represent a tumour-suppressor mechanism. In fibroblasts subjected to oxidative stress, Cav-1 has emerged as promoter of cell senescence [199–201] in virtue of its ability to sequester MDM2 [202], a negative regulator of p53 activity, or inhibit the activity of anti-oxidant enzymes, such as thioredoxin reductase 1 [203]. These findings suggest an investigation on the potential role of Cav-1 in controlling the p53 pathway in RMS cells.

Cav-1 and multiple control of receptorial systems for growth factors

Different receptorial systems for growth factors have been shown to signal aberrantly in RMS cells, including those for IGF-1R [148–150], EGFR [151, 152], PDGFR [150, 153] and VEGFR [154–156].

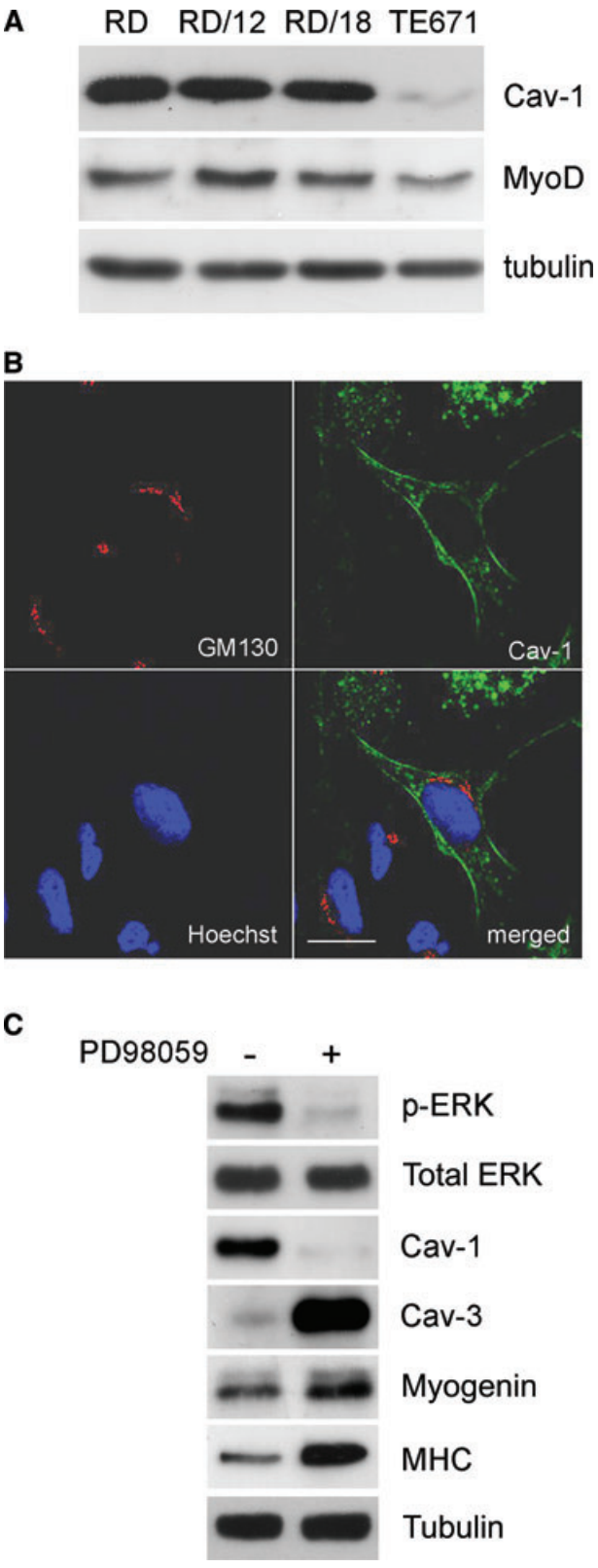

Fig. 3 Expression of caveolins in RMS cell lines. (**A**) Western blot analyses showing the expression of Cav-1 in several MyoD-positive ERMS cell lines. Tubulin was used as loading control. (**B**) As shown by confocal microscopy analysis, Cav-1 localizes at the plasma membrane or in intracellular vesicles of embryonal RD cells. GM130 marker was employed to stain the Golgi apparatus. Bars = 100 μm. (**C**) Ten microliters of PD98059 administration attenuates the ERK phosphorylation in RD cells and allows the transition from proliferation to differentiation, leading to Cav-1 down-regulation and increase of myogenin, MHC and Cav-3. Tubulin was used as loading control.

Table 2 The table summarizes the physiological role exerted by Cav-1 on different pathways which are involved in RMS

Pathway	Physiological role of Cav-1	Alterations linked to RMS
p53	Cav-1 may positively regulate p53 tumour suppressor function by sequestering MDM2 [202].	Germline p53 mutations predispose to different tumours (Li–Fraumeni syndrome), including RMS [141].
		Overexpression of MDM2, a p53 binding protein, is associated to ARMS tumours and cell lines [144, 165].
IGF1-R	Cav-1 is a positive regulator of IGF-IR signalling pathway [204–207].	Alterations in IGF1-R signalling are involved in RMS [148–150].
EGFR	Cav-1 inhibits the autophosphorylation of the EGF-R kinase *in vitro* [208].	HER1/EGFR is mainly expressed in ERMS, HER-2/EGFR in ARMS [151, 152].
PDGFR	Cav-1 inhibits the autophosphorylation of PDGF receptors in a dose-dependent manner [209].	Both ERMS and ARMS overexpress PDGFR-A and its ligands PDGF-C and PDGF-A [150, 153].
VEGFR	Cav-1 acts as a negative regulator of VEGFR-2 activity in endothelial caveolae [210].	Autocrine VEGF secretion stimulates RMS cell growth [154–156].
RAS/ERK	Cav-1 limits the RAS/ERK pathway activation in several cell types [53, 59, 211], including muscle satellite cells [10].	Activating *RAS* mutations are detected in ERMS cell lines [122–124, 196] and favour ERMS tumours [177, 181, 183, 190–193].
TGF-β/ myostatin	Cav-1 interacts with TGF-β type I receptors (ALK receptors) to limit the activation of TGF-β pathways [213].	TGF-β superfamily members, such as TGF-β and myostatin, may impair RMS differentiation [214–218].
HGF/cMET	Cav-1 is a downstream target of the HGF/cMET signalling axis in muscle satellite cells [10].	Transgenic mice overexpressing HGF develop cancer, including RMS [146, 147].
		cMET is frequently overexpressed in RMS [160].
RAGE	Cav-1 is a downstream target of RAGE-mediated Src activation in Schwann cells [227] and endothelial smooth muscle cells [228].	Low RAGE expression in myoblasts and RMS cells correlates with increased proliferation, migration, invasiveness and tumour growth [229, 230].

Remarkably, Cav-1 exerts a multiple physiological control on several components of these pathways through multiple mechanisms, such as physical interaction, regulation of receptor phosphorylation and internalization. In particular, Cav-1 configures as a positive regulator of IGF-1R pathways [204–207], although it is a negative regulator of EGFR [208], PDGFR [209] and VEGFR-2 activities [210]. Therefore, the ability of these receptorial systems to generate downstream signalling may be influenced, at least in part, by deregulated expression and/or localization of Cav-1, leading to a significant change in the biological outcome promoted by each pathway.

Cav-1 and RAS/ERK signalling

Overactivation of RAS/ERK signalling is one of the factors predisposing to RMS [122–124, 177, 181, 106]. Historically, Cav-1 configures as a strong inhibitor of this pathway in several cell types and tissues [10, 53, 59, 211]. Actually, the elevated Cav-1 expression detected in human RMS embryonal RD cells has been correlated to hyperactivation of the ERK pathway (Fig. 3C) [23]. These findings indicate that, at least in RD cells, Cav-1 seems to be aberrantly converted to a downstream target of the ERK pathway, giving rise to an ambiguous situation, in which cell proliferation is associated to persistent Cav-1 expression, suggesting that Cav-1 has lost the ability to antagonize the RAS/ERK signalling. The main hypotheses explaining this behaviour could be referred to an impaired activity of additional proteins which might cooperate with Cav-1 in the inhibition of the RAS/ERK pathway; alternatively, the antagonistic role of Cav-1 on the ERK pathway might occur upstream *RAS*, thus rendering ineffective its inhibition in the presence of activating *RAS* mutations. In this regard, it is known that hyperactivation of the ERK pathway cooperates to shift the function of some proteins, as occurs for Sprouty-1, which changes its function from antagonist to agonist of the RAS/ERK pathway in ERMS tumours harbouring activating *RAS* mutations [212]. Thus, it is important to establish how the association between high Cav-1 expression and elevated RAS/ERK pathway might influence RMS cell behaviour.

Caveolins and TGF-β/myostatin signalling

Caveolins are long known to limit the TGF-β pathway [213], which is supposed to play a not less important role in RMS [214–218].

Transforming growth factor-β (TGF-β) signalling pathways regulate numerous physiological and pathological processes [219–221] and proceed from the cell membrane to the nucleus through the cooperation of the types I and II serine/threonine kinase receptors (TβR-I and -II) and their downstream SMAD effectors [222]. Different TGF-β ligands, such as TGF-β [214] and myostatin [223], have been implicated in poor differentiation of RD cells [214–218]. Remarkably, either Cav-1 or Cav-3 interacts with and inhibits the activity of TβR-I receptors at the membrane [97, 213, 224], thereby limiting the downstream signalling. In view of these findings, the expression of caveolins may influence the cell behaviour of RMS cells in response to TGF-β/myostatin ligands.

Cav-1 and HGF/cMET signalling

Elevated HGF/cMET signalling has been implicated in RMS [146, 147, 160] as well as in different tumours [225]. In particular, cMET overexpression plays a significant role in RMS maintenance, as its silencing reduces cell invasiveness and tumour growth in a model of RMS xenograft [160]. In muscle satellite cells, HGF/cMET signalling axis plays a major role to elicit the transcriptional suppression of Cav-1 during muscle regeneration, a mechanism which is supposed to be central for the repair of the injured site [10]. Hence, these findings suggest that the aberrant HGF/cMET signalling observed in RMS cells might possibly reflect on the expression levels of Cav-1.

Caveolins and RAGE signalling

Receptor for advanced glycation end-products (RAGE) is a multiligand receptor of the immunoglobulin superfamily with a positive or negative role in cancer progression and metastasis depending on the tumour type [226]. A role of RAGE in regulating Cav-1 and Cav-3 expression/phosphorylation has been reported in several cell types. Indeed, (i) RAGE-mediated Src activation induces Cav-1 phosphorylation in Schwann cells [227] and endothelial smooth muscle cells [228]; and (ii) RAGE engagement induces Cav-3 expression in a myoblast cell line, whereas functional inactivation of RAGE in normal myoblasts results in the acquisition of a tumour behaviour with concomitant reduced expression of Cav-3 [229]. In RMS cells, RAGE activity is predictive of reduced proliferation, invasiveness and increased differentiation [230]. These findings suggest that RAGE signalling might have a role in regulating caveolins expression in RMS cells.

Conclusions

This review outlines the great amount of data indicating that caveolins play an important role in tumour development and progression. In RMS, the most frequent childhood soft-tissue sarcomas sharing myogenic features, Cav-1 and Cav-3 configure as markers of immature or mature tumours, respectively. Hence, immunohistochemical detection of caveolins in conjunction with strengthened myogenic markers may provide a useful diagnostic tool for establishing more accurately the grading in tumour samples. In this regard, the availability of novel molecular signatures may be critical to discriminate particular RMS variants and implement the current classification. Two major criteria support the demand to establish the role of caveolins in RMS tumour progression. First, caveolins are scaffolding proteins regulating several targets and pathways which are central to RMS development, and therefore loss or gain of caveolins function may generate multiple effects on the tumour behaviour. Finally, Cav-1 represents a tumour conditional gene in cancer and, thereby, recognizing its precise role in RMS progression will be crucial to elaborate targeted therapies. In summary, this review open up new interesting perspectives for investigating the role of caveolins in a large variety of pathological mechanisms predisposing to RMS.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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