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# Rhabdomyosarcomas: an overview on the experimental animal models

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## Abstract

Rhabdomyosarcomas (RMS) are aggressive childhood soft-tissue malignancies deriving from mesenchymal progenitors that are committed to muscle-specific lineages. Despite the histopathological signatures associated with three main histological variants, termed embryonal, alveolar and pleomorphic, a plethora of genetic and molecular changes are recognized in RMS. Over the years, exposure to carcinogens or ionizing radiations and gene-targeting approaches *in vivo* have greatly contributed to disclose some of the mechanisms underlying RMS onset. In this review, we describe the principal distinct features associated with RMS variants and focus on the current available experimental animal models to point out the molecular determinants cooperating with RMS development and progression.

**Keywords:** rhabdomyosarcomas • animal models • skeletal muscle

## Introduction

Rhabdomyosarcomas have an incidence of about 50% of all soft-tissue sarcomas and 10% of all malignant solid tumours in children [1, 2]. They derive from mesenchimal progenitors committed to myogenic lineages and so may arise in almost any body district [3–6], thus exhibiting a muscle-specific expression pattern that makes this malignancy rather unique [7]. Diagnosis of RMS, indeed, is predicted by the immunohistochemical or molecular detection of Myogenic Regulatory Factors, such as MyoD and myogenin [8, 9], whereas the expression of contractile proteins, like myosin, is indicative of differentiated tumour phenotypes [10]. Basically, several different genomic imbalances and translocations have been recognized in RMS, leading to identification of a rather complex number of deregulated pathways and targets [3–5]. Recently, much attention has been devoted to the tumour-initiating cells involved in RMS development, suggesting that differences in tumour histology may be dependent on the presence of specific genetic changes in different mesenchymal cell progenitors [11–15]. In this article, we present an overview of the chemical, physical and genetic approaches employed to trigger RMS formation in different mammalian and non-mammalian models.

## Histological, genetic and molecular characteristics of RMS

RMS have been classified on the basis of histopathological criteria and genetic signatures. They include two major histological variants, termed embryonal (ERMS) and alveolar (ARMS), and a less common pleomorphic (PRMS) variant. ERMS are more responsive to treatments and make up to 80% of RMS in children of less

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than 10 yrs of age. ERMS may occur in any body district and are heterogeneous in terms of histological appearance, ranging from poorly to highly differentiated lesions, virtually resembling the multi-step process of embryonic muscle differentiation. ARMS, instead, are characterized by poorer prognosis and are mainly detected in the trunk and body extremities in adolescents and adults [16]. ARMS cells resemble lung alveoli, with clusters of eosinophilic tumour cells arranged loosely and disposed in an alveolar pattern. PRMS are rare and mainly found in adults, and typically have a poor clinical outcome [17, 18].

Rhabdomyosarcomas histotype is preferentially correlated with particular genomic aberrations (Table 1); in particular, ERMS are characterized by a severe genomic instability primarily due to losses or gains on different chromosomes [19–22]. The most frequent signatures characterizing ERMS are the loss of heterozygosis (LOH) and imprinting (LOI) on chromosome region 11p15.5 [23–29]. These genetic alterations trigger the impaired expression of different putative tumour suppressor genes like *H19* [30], *CDKN1C* (*p57/KIP2*) [31, 32], and *SLC22AIL* (*BWR1A*) [33], but also the overexpression of *IGF-2*, a tumour-promoting gene imprinted in the opposite direction [24, 34]. In addition, frequently associated with ERMS are the deficiency in Patched (*PTCH*) gene due to LOH on chromosome 9q22 [21, 35, 36] and activating mutations in *RAS* gene [37–39]. Alveolar, instead, are predominantly characterized by the presence of non-random chromosomal translocations [40–42], as well as by other less frequent genetic changes [43–49]. In particular, the t(2;13)(q35;q14) and t(1;13)(p36;q14) translocations account for about 70% and 10% of ARMS, respectively, giving 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 (FoxO1a) [40–42]. The so-originated Pax3-Fkhr and Pax7-Fkhr transcription factors enable an aberrant transcriptional programme, contributing to RMS progression through multiple mechanisms [19, 50–52]. Finally, in PRMS, a miscellaneous of several different genetic aberrations has been detected [53, 54].

Among the several molecular alterations found in RMS, some are frequent and others are rare, unveiling heterogeneous aetiologies under the convergent phenotype. As summarized in Table 2, the network of these alterations encompasses the expression of the chimeric Pax3- and Pax7-Fkhr factors [40–42] and the loss or gain of activity of different players, including members of the p53 [55–61], Rb [62] and CDKs families [31, 32, 44, 49], tumour-suppressor genes [30, 33], autocrine/paracrine growth factors [24, 34, 63–85], chemokines [86, 87], immunoglobulin superfamily members [88], myogenic proteins [50, 52, 89–92], and components of the Akt [93], n- and c-Myc [46–48, 94], Ras/Erk [37–39] and Sonic hedgehog [21, 35] pathways. Moreover, the involvement of certain gene aberrations has been inferred from the study of different human syndromes particularly correlated with RMS [59] (Table 3), including the Li-Fraumeni syndrome [55], Beckwith-Wiedemann syndrome [95], neurofibromatosis-1 [96], Costello syndrome [97], Gorlin syndrome [98], retinoblastoma [99], mosaic variegated aneuploidy syndrome [100], mismatch repair deficiency syndrome [101] and Rubinstein-Taybi syndrome [102].

Collectively, the large body of experimental evidence indicates that RMS development frequently requires the suppression of the p53 pathway in conjunction with secondary cooperating events, including the aberrant activity of different tyrosine-kinases receptors along the Ras axis, or the occurrence of Pax3/7-Fkhr chimeric products in the case of ARMS [3–6].

# Experimental animal models of RMS

Rhabdomyosarcomas development has been detected upon exposure to carcinogen agents or ionizing radiations (Table 4), as well as in several different genetically engineered animal models (Table 5). An overview of these models, together with their main pathological features, is provided below.

## Carcinogen agents and ionizing radiations-exposed animal models

### Heavy metals

Heavy metals (such as As, Cd, Cr, Ni, Co, Cu, Fe, Hg, Pb, Pt) represent an important family of highly toxic environmental pollutants arising as industrial by-products and displaying mutagenic and carcinogenic potential. Indeed, metal cations can catalyse the production of reactive oxygen species which, in turn, elicit a variety of macromolecular alterations by impairing cellular functions. About 50 years ago, RMS formation was first observed upon intramuscular injection of nickel and cobalt compounds into adult rats [103, 104]. Since then, other works have shown that nickel compounds are efficient triggers of RMS formation in rat and rabbit models [105–107]. More recently, an *in vitro* study revealed that cancerous cells derived from the nickel compounds-treated rats were characterized by a mature phenotype [108].

### Pyrrolizidine alkaloids

The pyrrolizidine alkaloid monocrotaline and its major metabolite dehydroretronecine are naturally occurring toxins widely distributed in the world. These alkaloids are among the most common poisonous plants affecting livestock, wildlife and humans, as they cause liver toxicity and cancer [109, 110]. Administration of dehydroretronecine produced RMS in over 50% of the treated rats, in addition to other neoplasms occurring at lower percentage such as myelogenous leukaemias, hepatocellular carcinomas, and pulmonary adenomas [111].

### Benzenediazonium sulphate and relative compounds

Benzenediazonium sulphate (BD) is formed during the cytochrome P-450 catalysed metabolism of the carcinogenic 1-(phenylazo)-2-hydroxynaphthalene (Sudan I, Solvent Yellow 14), which was used as a colouring agent for food and other materials in several countries. Furthermore, BD is a metabolic breakdown product of different classes

**Table 1** Genetic signatures associated with RMS histotypes

| | ERMS | ARMS | PRMS |
|---|---|---|---|
| LOH and/or LOI | 11p15.5 [23–29]<br>9q22 [21, 35, 36] | | |
| Translocations | 11p1-q11 [22] | t(2;13)(q35;q14)<br>t(1;13)(p36;q14) [40–42] | |
| Amplifications | 12q13-15 [20] | 12q13-15 [43–45]<br>2p24 [43, 46–48]<br>2q34-qter<br>15q24-26<br>1p36<br>13q31-32<br>1q21<br>8q13-21 [43] | 1q25-q31<br>11q13.5-q14<br>8p11.2-p11.1 [53] |
| Gains of chromosomes | 2, 7, 8, 11, 12, 13q21,<br>17, 18, 19, 20 [20, 21] | 13 [43] | 1p22-p23<br>7p<br>20/20p<br>1q21-q25<br>3p12<br>3q26-pter<br>4q28-q31 [54]<br>18/18p<br>8q21-q23/8q<br>22q [53, 54]<br>5, 6q [53] |
| Losses of chromosomes | 3, 6, 10, 14, 15, 16, 17 [20, 21]<br>9q22, 1p35-36, 14q21-q32 [21] | 16q<br>9q32-34<br>13q14-qter [43]<br>17p<br>9p21 [49] | 1q, 14, 17p<br>12q13.2-q13.3 [53]<br>10q23<br>15q21-q22<br>2q21-q35 [54]<br>3p<br>5q32-qter<br>13 [53, 54] |

of nitrogen-nitrogen bond-containing chemicals. Subcutaneous injections of BD in Swiss mice gave rise to different neoplasms that were classified as RMS, fibrosarcomas and osteosarcomas [112]. Other variants of this molecule, namely 4-hydroxy- and 4-methyl-BD, are two ingredients derived from the non-cultivable unedible *Agaricus xanthodermus* and the cultivable *Agaricus bisporus* mushrooms, respectively. These molecules promoted RMS development in mice, in addition to fibromas, fibrosarcomas and myxosarcomas [113, 114].

### *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine

*N*-nitroso compounds, such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), are chemical carcinogens detectable in preserved foodstuffs and cigarettes and represent important risk factors contributing to development of nasopharyngeal carcinoma [115]. It has been shown that fish models, such as Medaka (*Oryzias latipes*) and Zebrafish (*Danio rerio*), develop a broad range of neoplasms of mesenchymal derivation, including RMS, when exposed to MNNG [116, 117].

### Azoxymethane and methylazoxymethanol

Azoxymethane (AOM) is a potent carcinogen causing a high incidence of colon cancer in rodents [118]. Once administered, AOM is metabolized into methylazoxymethanol [119], a mitotoxic molecule that spontaneously decomposes to a reactive alkylating agent with tumourigenic [120] and neurotoxic properties [121]. Different fish species exposed to methylazoxymethanol-acetate exhibited RMS formation [122].

### Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons are implicated in the aetiology of human cancer due to exposure to cigarette smoke, urban air, pollution, coal combustion and certain occupational situations [123]. For example, exposure to 7,12-dimethylbenz[a]anthracene elicited RMS formation in Zebrafish, although at low incidence [124], whereas the same molecule was recently shown more effective in causing RMS in male Sprague–Dawley rats [125]. Also, RMS was detected in mice treated with benzo[a]pyrene

**Table 2** Principal molecular alterations detected in RMS

| Gene family | Molecular target | Alteration in RMS | References |
|---|---|---|---|
| Chimeric fusion genes | *PAX3-FKHR*<br>*PAX7-FKHR* | Product of translocation t(2;13)(q35;q14)<br>Product of translocation t(1;13)(q36,q14) | [40–42] |
| *P53*, *RB* and *CDKs* | *P53* | Loss of activity due to several different gene mutations and/or deletions | [55–59] |
| | *MDM2* | Overexpression and/or gene amplification | [58, 60] |
| | *P63, P73* | Transcript overexpression | [61] |
| | *RB* | Homozygous deletion on the protein-binding pocket domain | [62] |
| | *CDKN2A, ARF* | Gene deletion | [49] |
| | *CDKN2B* | Gene deletion | [49] |
| | *CDK4* | Overexpression due to locus amplification in 12q13-15 | [44] |
| | *CDKN1C (p57/KIP2)* | Loss of expression due to LOH in 11p15.5 locus | [31, 32] |
| Tumour-suppressor genes | *H19* | Loss of expression due to LOH in 11p15.5 locus | [30] |
| | *SLC22AIL (BWR1A)* | Loss of expression due to LOH in 11p15.15 locus | [33] |
| Autocrine/paracrine growth factors | *HGF*/*c-MET* | *c-MET* activating mutations<br>*c-MET* overexpression and/or amplification<br>Pax-Fkhr-dependent *c-MET* overexpression | [63–68] |
| | *IGF1R* | Pax-Fkhr-dependent *IGF1R* up-regulation | [69–71] |
| | *IGF-2* | Overexpression due to LOI, LOH and paternal disomy<br>Pax3-Fkhr-dependent overexpression | [24, 34] |
| | *IGFBP5* | Overexpression | [72, 73] |
| | *HER-1/EGFR* | Overexpression | [74] |
| | *HER-2* | Overexpression | [74] |
| | *PDGFR-A*, *PDGF*-A and *C* | Overexpression | [75] |
| | *VEGF* | Overexpression of both short and long isoforms | [76] |
| | *VEGFR1* | Overexpression at mRNA and protein levels<br>Pax3-Fkhr-dependent overexpression | [76–78] |
| | *FGFR4* | High expression at mRNA and protein levels<br>Activating mutations in the tyrosine-kinase domain | [79] |
| | *FGF, glypican-5* | Gene amplification | [80] |
| | NGF pathway | Anti-apoptotic autocrine loop | [81] |
| | TGF-β/myostatin | Increased expression | [82–85] |
| Chemokines | *MMP2*, *CXCR4* | Pax3-Fkhr-dependent overexpression | [86] |
| | *IL-4R* | Pax3-Fkhr-dependent overexpression | [87] |
| Immunoglobulin superfamily | *RAGE* | Reduced gene expression | [88] |
| Myogenic proteins | MyoD | Frequent expression in inactivated form | [50, 52, 89–92] |

**Table 2** Continued

| Gene family | Molecular target | Alteration in RMS | References |
|---|---|---|---|
| *AKT* | Akt | High levels of phosphorylated Akt (Thr308 and Ser473) | [93] |
| *RAS*/*ERK* and *MYC* | *KRAS-2, NRAS* | Activating point mutations | [37] |
| | *HRAS-1* | Activating point mutations | [38, 39] |
| | *n-MYC* | Gene amplification | [46–48] |
| | *c-MYC* | Overexpression | [94] |
| Sonic hedgehog | *PTCH1* | Gene deletion | [21, 35] |

**Table 3** Human syndromes associated with RMS

| Human cancer syndromes | Locus | Genetic mutation | MIM ID | References |
|---|---|---|---|---|
| Li-Fraumeni syndrome | 17p13.1, 9p21 | Germline transmission of a mutated *P53* allele | 151623 | [55] |
| Beckwith-Wiedemann syndrome | 11p15.5 | Mutation or deletion of imprinted genes within the 11p15.5 locus | 130650 | [95] |
| Neurofibromatosis-1 | 17q11.2 | Mutation in *NF1* | 162200 | [96] |
| Costello syndrome | 11p15.5 | Germline mutation in *HRAS* | 218040 | [97] |
| Gorlin syndrome | 9q22.3 | Germline mutations in *PTCH1* or *PTCH2* | 109400 | [98] |
| Retinoblastoma | 13q14.1-q14.2 | Germline mutation in *RB1* | 180200 | [99] |
| Mosaic variegated aneuploidy syndrome | 15q15 | Constitutional biallelic truncating and missense mutations in *BUB1B* | 257300 | [100] |
| Mismatch repair deficiency syndrome | 7p22, 3p21.3, 2p16, 2p22-p21 | Biallelic germline mutations of *MLH1*, *MSH2*, *MSH6* or *PMS2* | 276300 | [101] |
| Rubinstein-Taybi syndrome | Unknown | Mutations in *CREBBP* (>60%) or *EP300* (about 3%) | 180849 | [102] |

MIM (Mendelian Inheritance in Man) identification numbers referred to each syndrome can be used to retrieve further information at the following site: http://www.ncbi.nlm.nih.gov/omim.

[126], even in association with nickel compounds [105]. Interestingly, benzo[a]pyrene carcinogenicity was lost in mice lacking the aryl hydrocarbon receptor [127].

### Depleted uranium and lead

An interesting study was recently performed to assess the health risk effects of soldiers exposed to depleted uranium and lead employed to build tungsten alloy-based munitions [128]. For this purpose, male F344 rats were implanted intramuscularly with pellets of weapons-grade alloy to simulate shrapnel wounds. Within 4–5 months from the time of implantation, animals exhibited serious haematological changes indicative of polycythemia and aggressive PRMS in the surrounding of the pellets, with rapid formation of lung metastases.

### Ionizing radiations

Epidemiological evidence identifies ionizing radiations as causative agents contributing to the stepwise process of carcinogenesis. Indeed, repeated doses of β-radiation on the backs of CD-1 mice triggered a subset of neoplasms, with RMS being the most frequently observed [129]. RMS cell lines derived from these tumours all exhibited p53 inactivating mutations, suggesting that excessive exposure to radiation contributes to RMS development through the loss of p53 tumour-suppressor activity.

**Table 4** Experimental animal models of RMS – chemical and physical triggers

| Triggers | Modality of administration and animal models | Tumour analysis | References |
|---|---|---|---|
| Heavy metals: nickel and cobalt compounds | Intramuscular single injection in thigh of Wistar rats, C3H or Swiss mice | PRMS, highly anaplastic RMS, fibrosarcoma and myoma | [103–105] |
| | Testis injection in Fisher rats | RMS, fibrous histiocytomas and fibromas | [106] |
| | Intramuscular implant in rabbits | RMS with small polygonal or elongated cells, mature myofibres | [107] |
| Pyrrolizidine alkaloids: dehydroretronecine or monocrotaline | Subcutaneous injection in male Sprague-Dawley rats | RMS | [111] |
| Benzenediazonium sulphate and derivates | Subcutaneous injection in Swiss mice | RMS, fibrosarcomas, osteosarcoma, fibromas and myxosarcomas | [112–114] |
| *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine | Microinjection, water or dietary exposure in Medaka or Zebrafish | RMS and other mesenchymal-derived sarcomas | [116, 117] |
| Azoxymethane and methylazoxymethanol acetate | Water exposure in Medaka and Guppy fish | RMS | [122] |
| Polycyclic aromatic hydrocarbons: dimethylbenz[a]anthracene and benzo[a]pyrene | Microinjection, water or dietary exposure in Zebrafish | RMS in embryos and juveniles | [124] |
| | Subcutaneous injection in neonatal male Sprague-Dawley rats | PRMS and ERMS | [125] |
| | Subcutaneous implantation of filters overlaid with gelatine containing benzo[a]pyrene in mice | Foreign-body-induced sarcoma and RMS | [126] |
| Tungsten alloy-based munitions embedded with uranium and lead | Intramuscular leg implantation of nickel- and tantalum-pellets in male F344 rats | Polycythemia and PRMS with lung metastases | [128] |
| Ionizing radiations | Repeated doses of β-radiation in CD-1 mice | RMS, squamous-cell carcinoma and malignant fibrous histiocytoma | [129] |

## Virus infection and transgenic expression of viral proteins

Results from a study performed in the 1968s have shown that the infection of newborn rats with Moloney-murine sarcoma virus (MoMSV) predisposes to RMS formation [130]. After virus inoculation in the inguinal area, the solid tumour grown rapidly and expanded toward the leg muscles and the dorsal musculature. Metastases occurred regularly in the lungs and draining lymph nodes, and the metastatic cells in the lung closely resembled the cells of the primary tumour. A subsequent *in vitro* analysis demonstrated that RMS cells derived from the MoMSV-infected rats had an immature phenotype, displaying staining for desmin, but lack of myosin expression [108].

More recently, transgenic mice harbouring the Simian Virus T Antigene (*SV40 TAg*) gene under the control of the beta-globin control region were generated, attempting the possibility to observe haematopoietic malignancies as a consequence of the erythroid-specific expression [131]. Unlikely, these mice developed PRMS in different anatomic sites and showed hyperplasia of the pancreatic islet cells, which progressed to pancreatic islet tumour.

In another model, transgenic mice expressing the 2.7-kb *SV40 TAg* early region under the control of the 5′ region of the *SM22alpha* gene (expressed in embryonic cardiac muscle) developed a rare form of cardiac RMS at the age of approximately 8–12 weeks [132]. Authors reasoned that the SV40 TAg protein and/or its upstream regulatory region may be implicated in the binding and sequestration of specific protein partners whose identity was yet unknown and whose loss of function may be involved in RMS formation. To reconcile these data with some recent findings, it is now accepted that SV40 TAg, like other viruses products,

**Table 5** Experimental animal models of RMS – biological triggers

| Triggers | Treatment or genetic approach and animal model | Tumour analysis | References |
|---|---|---|---|
| Virus and viral proteins | MoMSV inoculation in newborn Wistar rats | Undifferentiated RMS with lung and limph node metastases | [130] |
| | Erythroid-specific transgenic expression of *SV40 Tag* in SJL mice | PRMS and pancreatic islet tumour | [131] |
| | Cardiac-specific transgenic expression of *SV40 TAg* in C57BL/6 mice | Cardiac RMS | [132] |
| *P53* | Knock-out *P53* in C57BL/6 × CBA mice | Undifferentiated RMS and other neoplasms | [135] |
| | Double knock-out *P53* and *FOS* in 129Sv X C57BL/6 mice | ERMS | [139] |
| | Transgenic *HER-2/neu* expression in Balb/c mice with $P53^{+/-}$ background | ERMS | [143] |
| | $KRAS^{G12V}$ conditional expression in adult Balb/c mice with $P53^{+/-}$ or $P53^{-/-}$ background | PRMS | [144] |
| | Transgenic $KRAS^{G12V}$ expression and simultaneous loss of P53 or gain of $P53^{R172H}$ mutant in C57B16J/S129 mice | PRMS with lung metastases | [145] |
| *PAX3-FKHR* | *PAX3-FKHR* knock-in expression and *P53* or *INK4a/ARF* knock-out expression in conditional mice | ARMS | [149] |
| *RAS* | $KRAS^{G12D}$ expression alone or combined with a $P53^{+/-}$ or $P53^{-/-}$ background in Zebrafish | Highly invasive ERMS | [158] |
| *HGF* | Transgenic broad overexpression of *HGF* in FVB/N mice | RMS, amelanotic melanoma, hepatic and mammary tumours | [64] |
| | Transgenic *HGF* expression in FVB/C57BL/6 mice with a background *INK4a* deficient | Lymphomas, fibrosarcomas and multifocal ERMS | [68] |
| Sonic hedgehog | $PTCH^{+/-}$ knock-out in CD-1 mice | RMS | [36] |
| | Conditional $PTCH^{+/-}$ knock-out in C57BL/6 × CD-1 mice | RMS | [174] |
| | Conditional $P53^{-/-}$ in Balb/c mice with $PTCH^{+/-}$ background | ERMS | [175] |
| | Gene-trap mediated $SUFU^{+/-}$ in C57BL/6 mice with $P53^{-/-}$background | RMS | [176] |
| Muscular disorders-associated proteins | Nonsense mutation in dystrophin in non-transgenic mdx mice (model of DMD) | ARMS and ERMS | [185, 186] |
| | Mdx mice interbreeded with *P53*-deficient mice | ERMS | [191] |
| | Knock-out $\alpha$*SGCA* in C57BL/10ScSn/J mice (model of LGMD-2D) | ERMS | [186] |
| | Deficiency of dysferlin in A/J mice (model of LGMD-2B) | PRMS at high frequency | [192] |

has the ability to bind p53 and determine its loss of function, as recently demonstrated in patients affected by Li-Fraumeni syndrome with one *P53* allele active [133].

Thus, these findings suggest that viral proteins such as SV40 TAg, having the ability to bind proteins like p53 and pRb, may contribute to RMS pathogenesis.

## Gene-targeted animal models

### P53 pathway

*P53* tumour-suppressor activity promotes apoptosis, senescence or reversible protective cell cycle arrest upon a variety of cellular damage signals [134]. In this sense, cells harbouring inactivating *P53* gene mutations are predisposed to cancer, as they escape self-protecting cell death and acquire a long-lived resistant condition. Patients affected by the Li-Fraumeni syndrome, harbouring germline *P53* mutations, develop soft-tissue sarcomas [55], including a significant percentage of RMS [13,56]. In addition, *P53* mutations and/or overexpression of its negative regulator *MDM2* are frequently recognized in RMS [58, 60]. So far, *P53* null mice were generated through homologous recombination strategy [135]. These mice had normal development, but were susceptible to spontaneous formation of different cancers, including RMS at low incidence [135–138]. Subsequent works have firmly supported that RMS incidence is greatly increased when loss of p53 activity occurs in association with other deregulated pathways. As such, RMS tumour frequency was increased in mice upon concomitant loss of *P53* and *FOS* [139], the latter being a major component of the AP-1 transcription factor, which regulates various biological processes by converting extracellular signals into changes in the expression of specific target genes [140]. Also, the activity of the tyrosine-kinase HER-2/neu receptor, which is expressed in approximately one-half of human RMS [141] and is involved in the transformation of many cell types [142], promoted ERMS formation in transgenic mice when coupled to loss of *P53* [143].

A synergism between p53 and Ras pathways has been frequently observed in RMS. For instance, the conditional expression of the cancer-related activating $KRAS^{G12V}$ mutation in adult muscles of *P53* null mice triggered formation of PRMS [144], whereas the same $KRAS^{G12V}$ form in the presence of the $P53^{R172H}$ mutant triggered PRMS with more aggressive metastases [145], suggesting that p53 mutants, due to gain of toxicity, can have more deleterious effects on tumour development compared with the sole p53 loss [146].

In summary, loss of p53 activity and/or gain of p53 cytotoxic function play(s) a central role in RMS development, especially when coupled to the aberrant activity of additional pathways.

### Pax3-Fkhr transcription factor

Pax3-Fkhr chimeric factor strongly cooperates together with the loss of *P53* or *INK4a/ARF* locus in ARMS onset. Transgenic mice carrying *PAX3-FKHR* exhibited defects in muscle development, including ectopic skeletal myogenesis in the developing neural tube, although they did not exhibit spontaneous tumour formation [147, 148], supporting the idea that Pax3-Fkhr expression was not sufficient *per se* to cause RMS. However, targeting a conditional *PAX3-FKHR* knock-in allele in terminally differentiating Myf6-expressing myofibres promoted ARMS formation [149]. Strikingly, ARMS frequency was increased in these conditional mice by the simultaneous disruption of either p53 pathway or *INK4a/ARF* locus [14, 149], the latter containing two overlapping tumour suppressor genes, $p16^{INK4a}$ and $p14^{ARF}$ [68], involved in the regulation of cell cycle, senescence and apoptosis [150, 151]. These data suggest that expression of Pax3-Fkhr in differentiating Myf6-myofibres seems to be a *sine qua non* condition predisposing to ARMS, particularly when coupled to disruption of gene targets controlling cell cycle, such as *P53* and those included in the *INK4a/ARF* locus.

### Ras/Erk pathway

Activating *RAS* mutations have been primarily associated with ERMS [13, 37–39, 152–155]. Indeed, mutations in components of the Ras pathway are responsible of clinically overlapping dominant disorders that are characterized by RMS development, including the Noonan syndrome, Costello syndrome, cardiofaciocutaneous syndrome and LEOPARD syndrome [156]. Costello syndrome, in particular, is characterized by short stature, facial dysmorphism, cardiac defects and predisposition to cancers, including ERMS, because of germline activating mutations in the *HRAS* gene on chromosome 11p15.5 [97, 157]. Indeed, an elegant model of ERMS has been established by the delivery of a transgenic construct harbouring an activated RAS form ($KRAS^{G12D}$) in muscle-associated cells of Zebrafish [158]. Injected embryos developed highly invasive tumours composed of heterogeneous cell populations, comprising undifferentiated muscle cells, multi-nucleated striated muscle fibres, and infiltrating blood cells. In addition, tumour incidence markedly increased when the $KRAS^{G12D}$ transgene was injected into mutant fish with a $P53^{+/-}$ and $^{-/-}$ background, confirming the cooperation between Ras and p53 pathways in RMS development. On the basis of microarray analysis on sorted cell populations of Zebrafish, authors postulated that the tumour-initiating cells were reasonably similar to muscle satellite cells. In summary, oncogenic *RAS* may itself play a primary role in ERMS development, especially when the gain of activity occurs in muscle satellite cells. Moreover, RMS frequency is greatly increased upon the simultaneous loss of p53 function, as observed in Zebrafish [158] and adult mice [144, 145].

### Hgf/c-Met pathway

*C-MET* proto-oncogene encodes a tyrosine-kinase receptor that, upon binding with the hepatocyte growth factor (Hgf), promotes cellular growth, motility and survival, extracellular matrix degradation and angiogenesis [159, 160]. Excessive activation of this pathway has been implicated in a subset of human cancers, including RMS [161, 162]. In transgenic mice, inappropriate Hgf expression gave rise to distinct tumours of both mesenchymal and epithelial origin [64, 163, 164], with a prevalence of malignant mammary tumours, melanomas, RMS, fibrosarcomas, squamous papillomas, basal cell and hair follicle tumours. Later, it has been demonstrated that aberrant c-Met signalling and simultaneous *INK4a/ARF* locus inactivation are critical for RMS genesis [68]. Indeed, a consistent percentage of $INK4a/ARF^{-/-}$ mice developed lymphomas and fibrosarcomas [165], whereas almost all $INK4a/ARF^{-/-}$ mice overexpressing Hgf exhibited highly invasive RMS at 3 months of age [68]. These data suggest that constitutive activation of c-Met and simultaneous absence of $p16^{INK4a}$

and $p19^{ARF}$ may give rise to a pre-malignant population of myogenic precursors, which cannot withdraw from the cell cycle and are resistant to p53-mediated apoptosis [166, 167]. Unlike embryogenesis, during which a Pax3-dependent expression of *c-MET* in the lateral dermomyotome is required for the appropriate migration of myogenic precursors to the limb [168], an aberrant Pax3-Fkhr-dependent c-*MET* transcription takes place in RMS. As the Hgf/c-Met pathway is only transiently required for the activation of satellite cells following skeletal muscle injury [169], its persistent gain of function in RMS cells has been supposed to allow invasiveness through continuous proliferation and migration [63, 65–67], ideally resembling a regenerating muscle that fails to repair [68].

**Sonic hedgehog pathway**

Inappropriate activation of the Sonic hedgehog (Shh) pathway, due to Ptch1 receptor inactivation, has been associated with familial cancer. In particular, germline mutations in the *PTCH1* gene lead to Gorlin syndrome, also termed Nevoid Basal Cell Carcinoma Syndrome, characterized by a variety of clinical problems such as increased body size, developmental abnormalities of the skeleton, and increased incidence of sporadic Basal Cell Carcinomas (BCC), Medulloblastoma (MB) and RMS [98]. In addition, a deficiency in *PTCH1* gene due to LOH on chromosome 9q22 has been further implicated in RMS [21]. Ptch1 basally suppresses the activity of the seven-pass membrane protein Smoothened (Smo), while the binding to the Sonic hedgehog (Shh) relieves the inhibition of Smo, culminating in the activation of the downstream Gli transcription factors. The latter regulate a variety of processes in invertebrate and vertebrate embryonic development [170, 171]. So far, $PTCH1^{+/-}$ mice have been considered as a model of multi-organ tumourigenesis [172], as they were characterized by the development of many characteristic features of Gorlin syndrome, including a predisposition to radiation-induced teratogenesis and RMS formation [36]. Importantly, $PTCH1^{+/-}$mice displayed elevated Igf-2 levels [35, 36], suggesting that Ptch1 acts as a negative regulator of Igf-2, which is in turn required for the formation of MB and RMS. In comparison with $P53^{+/-}$ mice, $PTCH1^{+/-}$ mice predominantly showed less aggressive RMS due to a greater degree of differentiation [173], clearly highlighting how different mutations can have a different impact on tumour behaviour. Later on, the development of an elegant conditional mouse model allowed to demonstrate that the time-point and the gene dose of *PTCH1* inactivation predispose to development of certain tumours rather than others [174]. In particular, RMS was observed when *PTCH1* heterozygosity was induced prenatally [174], especially in the presence of simultaneous loss of p53 [175]. On the other hand, mono- or bi-allelic postnatal deletion of *PTCH1* respectively lead to hamartomatous gastrointestinal cystic tumours, BCC precancerous lesions of the gastrointestinal epithelium and mesenteric tumours [174].

Recently, mice deficient for another regulator of the Shh pathway, termed Suppressor of Fused (*SUFU*), have been generated [176]. *SUFU* is a negative modulator of Shh signalling [170] and its gene ablation results in embryonic lethality [177], suggesting a critical role in higher organisms. Like *PTCH1*, *SUFU* is believed to be a tumour-suppressor gene [178], as a subset of MB patients carry germline and somatic *SUFU* mutations. In comparison with $PTCH1^{+/-}$ mice, $SUFU^{+/-}$ mice were not tumour prone; however, simultaneous loss of *SUFU* and *P53* triggered MB and RMS in mice [176].

Overall, these data suggest that inappropriate activation of the Shh pathway contributes to RMS development, especially in association with p53 loss of function.

**Muscular disorders associated-proteins**

Four different animal models of neuromuscular disorders have been associated with RMS development. Among them, the non-transgenic mdx mice ideally represent the animal phenocopy of the X-linked Duchenne Muscular Dystrophy (DMD) [179], as they lack dystrophin due to a premature stop codon in exon 23 of dystrophin gene [180–182]. Dystrophin confers resistance to skeletal, cardiac and smooth muscle cells [183] by connecting F-actin in the subsarcolemmal cytoskeleton to the Dystrophin-Glycoprotein Complex (DGC) that spans the sarcolemma and attaches to laminin-2 (merosin) in the extracellular matrix [184]. In particular, it has been shown that old mdx mice (between 16.5 and 24 months of age) develop ARMS [185], although another research group has described development of ERMS in the same model [186]. Spontaneous formation of ERMS has been also detected in alpha sarcoglycan ($\alpha$*SGCA*) deficient mice [186], which represent the animal phenocopy of the autosomal recessive Limb-Girdle Muscular Dystrophy-2D form (LGMD-2D) [187, 188]. The $\alpha$*SGCA* gene encodes a transmembrane glycoprotein that stabilizes the DGC complex [184] and protects muscle cells from contraction-induced damage [189, 190]. It is worth noting that all mdx and $\alpha SGCA^{-/-}$ mice developing RMS were characterized by harbouring cancer-related mutated p53 forms or overexpressing mutated or deleted Mdm2 forms lacking the p53-binding domain (in the case of mdx model) [186], confirming that disruption of the p53 pathway cooperates with RMS formation. To further corroborate this evidence, *P53*-deficient mdx mice have been recently generated, thus demonstrating that the regenerative microenvironment in skeletal muscle of mdx mice, when coupled to *P53* deficiency, is sufficient to robustly induce ERMS in young mice [191]. Finally, development of PRMS at a high frequency has been detected in the A/J mouse strain [192], characterized by a progressive muscular dystrophy homologous to LGMD-2B due to lack of dysferlin, a protein involved in muscle repair [193, 194].

Results from studies on these animal models suggest that the continuous activation and proliferation of satellite cells, characterizing the lifelong myofibre degeneration and regeneration in muscular disorders, predispose a local environment that may greatly increase the chance of developing RMS, particularly in the presence of Mdm2/p53 cancer-associated alterations.

## Conclusions

Generation of animal models has provided a powerful tool for understanding the molecular determinants cooperating with RMS formation. Due to technology limitations, until few years ago, experimental

induction of RMS was merely obtained by exposure to disparate classes of chemicals and ionizing radiations as well. Over the last two decades, the growing availability of genetic models has clearly outlined that the onset of RMS, as commonly seen in different cancers, requires the simultaneous occurrence of multiple aberrant molecular events, such as the loss of *P53*, *RB* and *INK4a/ARF* function along with the gained activity of Hgf/c-Met, Ras, Shh pathways and Pax3/7-Fkhr chimeric factors. Recent data further confirmed that loss of skeletal muscle integrity, as observed in some neuromuscular disorders, may supply a local tissue environment predisposing to RMS development. Apart from these different cues, RMS tumour histotype seems to be developmentally stage-dependent, being dictated from the timing and cell host in which specific molecular alterations arise.

In perspective, it will be attractive to bring together old and new evidence by coupling chemical and physical exposure in gene-targeted animal models. This combined approach could help to unravel how a specific genetic background may predispose to or protect from cancer formation in the presence of environmental risk factors.

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## Conflict of interest

The authors confirm that there are no conflicts of interest.

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