HHS Public Access

Author manuscript

Crit Rev Oncog. Author manuscript; available in PMC 2017 June 27.

Published in final edited form as:

Crit Rev Oncog. 2015 ; 20(3-4): 227–243.

Pediatric Rhabdomyosarcoma

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Abstract

Rhabdomyosarcoma is the most common soft-tissue sarcoma of childhood, and despite clinical advances, subsets of these patients continue to suffer high levels of morbidity and mortality associated with their disease. Recent genetic and molecular characterization of these tumors using sophisticated genomics techniques, including next-generation sequencing experiments, has revealed multiple areas that can be exploited for new molecularly targeted therapies for this disease.

Keywords

Rhabdomyosarcoma; Alveolar Rhabdomyosarcoma; Embryonal Rhabdomyosarcoma; Targeted Therapy; Genomics; Epigenetics; Development

I. INTRODUCTION

Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma of childhood, with an annual incidence of 4.5 cases per 1 million children, making it the third most prevalent extracranial solid tumor of childhood after neuroblastoma and Wilms tumor.[1] RMS tumors typically are associated with the skeletal muscle lineage, and approximately 50% of cases are diagnosed in the first decade of life. RMS is currently categorized by histopathology into distinct subtypes, including embryonal, alveolar, pleomorphic, and sclerosing/spindle cell pathology, which have distinct molecular and clinical correlates.[2] In the 1970s and 1980s a multimodal chemotherapy backbone of vincristine, actinomycin, and cyclophosphamide was established as an effective treatment for RMS.[3] Through a series of collaborative group clinical trials, dose modification of this backbone, coupled with improvements in local control and supportive care, have led to impressive gains in survival over the past decades. Patients with low-risk disease now have a 5-year survival that approaches 90%, and current efforts are focused on dose reduction to avoid long-term effects.[4] Relapse-free survival for patients with localized disease has improved to 70–80%, albeit with significant toxicity.[4] Despite these impressive and important gains, several clinical trends have emerged. First,

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there has been a general flattening in the improvement in outcome for all patients. This is likely because, despite several randomized studies to evaluate dosing and schedule, the systemic chemotherapy backbone has remained largely unchanged since the 1970s. This effect is most pronounced for patients with intermediate and high-risk disease who, in a series of trials, experienced escalating dose intensification and compression of cytotoxic therapy with minimal gains.[5,6] Second, patients with high-risk disease or recurrent disease continue to suffer a dismal prognosis (5-year survival <30% and 17%, respectively).[5,7] Finally, clinical trials that integrate the growing knowledge of the oncogenic mechanisms of these tumors with novel therapies have been slow to emerge. This review summarizes recent advances in the understanding of the genetic and molecular basis of RMS and highlights how investigators and clinicians are using this information in an effort to improve outcomes for patients with RMS.

II. RMS GENETICS

The association of RMS with familial cancer syndromes, most notably Li-Fraumeni syndrome,[8] neurofibromatosis,[9] Beckwith-Wiedemann syndrome,[10] and Costello syndrome,[11] has made the genetics of RMS an area of intense study. Decades of targeted sequencing and microarray methods have led to the discovery of loss of heterozygosity at 11p15.5[12]; mutations in *TP53*,[13] *NRAS*, *KRAS*, *HRAS*,[14] *PIK3CA*, *CTNNB1*,[15] and *FGFR4*[16]; and the characteristic translocations involving the *PAX3* or *PAX7* genes with *FOXO1*[17] that have defined the genomic characteristics frequently associated with histologic and clinical features of this disease.

Several more recent large-scale, next-generation sequencing studies of primary RMS tumors have been reported.[18–20] These studies revealed in a comprehensive manner the landscape of mutations, copy number changes, and genomic rearrangements that define these tumors. These studies each show that primary RMS has a low overall mutation rate (0.31 protein-coding mutations/Mb) and is characterized by 2 distinct genotypes, which can clearly be defined by the presence or absence of a *PAX* gene rearrangement (Fig. 1). Next-generation sequencing studies have confirmed that RMS should not be solely diagnosed by histology but by the presence (fusion-positive RMS) or absence (fusion-negative RMS) of a *PAX3/7* gene fusion.[18]

A. *PAX* Fusion–Positive RMS

In RMS, the *PAX3* or *PAX7* gene fusions were originally found through physical mapping and cloning studies, which revealed the rearrangement of chromosome 2 or 1 in a reciprocal translocation with FOXO1, found on chromosome 13.[17,21] Follow-up studies have confirmed that juxtaposition of the N-terminus of the paired box genes with the C-terminus of the forkhead transcription factor characterizes a distinct subset of RMS genotypes. Other infrequent rearrangements of the *PAX3* gene also have been observed in tumors with alveolar histology, including the in-frame fusion with the nuclear receptor coactivator *NCOA1*,[22,23] or the chromatin remodeling gene *INO80D*.[18] The tumors that harbor these fusions retain the expression signature characteristic of the canonical fusions *PAX3-FOXO1*

and *PAX7-FOXO1* and define a subset of tumors previously described as fusion-negative alveolar histology.

In general, tumors that have a *PAX* gene translocation have an extremely low overall mutation rate (0.1 protein-coding mutation/Mb) and, interestingly, no recurring genes with single nucleotide mutations[18] (Fig. 2). While recurrent collaborating point mutations have not been found in these tumors, regions of focal genomic amplification are frequently observed (Table 1). Multiple genome-wide analyses of copy number alterations in RMS to date have been completed using the single nucleotide polymorphism array technology. The most commonly amplified genomic regions observed in *PAX* gene fusion–positive tumors are 2p24, containing the *MYCN* oncogene, and 12q13-q14, which includes *CDK4*.[26] The amplification of *MYCN*, which occurs in 28% of fusion-positive cases, has been confined to a genomic region less than 1 Mb, including the same region frequently observed in neuroblastoma cases.[27] This region includes only 2 genes (*MYCN* and *DDX1*) and is observed most commonly in *PAX3-FOXO1* fusion–positive RMS. While the number of cases remains small, no correlation between 2p24 amplification and RMS clinical outcome has been shown, in contrast to neuroblastoma.[25] Amplifications of 12q13-q14, however, have been associated with significantly worse failure-free and overall survival independent of *PAX* gene fusion status.[25] This amplicon also is observed in multiple other tumor types, including lung cancer, glioblastoma, and osteosarcoma. The observed region has been confined to a common region, 0.55 Mb in length, that contains 27 genes, including *CDK4*. Expression analysis confirms that the genomic amplification results in overexpression of *CDK4*. Other amplified regions in fusion-positive tumors include 15q24-26, 1p36, 13q31, 1q21, and 8q13-21.[26] The regions of 1p36, which encompasses the *PAX7* locus, and 13q31, which includes *MIR17HG*, are associated specifically with *PAX7-FOXO1* tumors.

B. Fusion–Negative RMS

In contrast to *PAX* fusion–positive samples, tumors that do not harbor the fusion are characterized by a more heterogeneous histology, complex karyotype, regions of loss of heterozygosity, and an increased presence of single nucleotide point mutations. These tumors display a wide range of causative mutations. The mutation most frequently occurs within one of the Ras genes (*NRAS* > *KRAS* > *HRAS*), a receptor tyrosine kinase (*FGFR4* ≫ *ERBB2*), or the catalytic component (*PIK3CA*) of the phosphoinositide-3 kinase (PI3K) complex. Other genes that are recurrently mutated include the ubiquitin ligase *FBXW7*, as well as *NF1*, *TP53*, *CTNNB1*, and the transcriptional repressor *BCOR*[28] (Table 2). In addition, 2 recent studies have identified that point mutation of the myogenic muscle differentiation transcription factor *MYOD1* defines an aggressive subset of embryonal RMS[20] and adult spindle cell RMS.[31]

Perhaps the most unifying feature of fusion-negative tumors is the loss of heterozygosity at the 11p15.5 locus,[12] which occurs in a majority of fusion-negative tumors and also is observed in the Beckwith-Wiedemann syndrome,[33] hepatoblastoma,[34] and Wilms tumor.[35] The observed allelic loss of the maternal allele is frequently associated with duplication of the remaining allele, resulting in paternal isodisomy.[36] While many of the genes involved in the region have been implicated in oncogenesis, *H19*, *IGF2*, and *CDKN1C* have been the

most extensively studied. Loss of imprinting at the *IGF2* gene locus is associated with massive overexpression of IGF2, which is a nearly universal finding in RMS.

Chromosome- and chromosome arm-level gains and losses are frequent events in fusion-negative tumors. Multiple array studies have reported recurrent gains of chromosomes 2, 7, 8, 12, and 13.[37–39] In addition, focal losses of 9q32-34, which includes *CDKN2A*, and 17p, which includes the *TP53* and *NF1* loci, are observed. One recurrent focal amplification event that occurs in fusion-negative tumors is the high copy gain of the 12q14-15 locus, containing the *MDM2* gene. Alteration of the *MDM2* locus is a common event in soft-tissue sarcomas,[32] and the gene product is known to bind and inactivate *TP53*. In RMS, the *MDM2* amplicon can overlap with the *CDK4* amplicon, but more frequently the 2 alterations seem to be mutually exclusive.

III. RMS EPIGENETICS

With the emergence of novel techniques to interrogate the epigenome, there have been efforts to define the DNA modifications that affect transcription within these tumors. Hypermethylation of 5′ regulatory regions of cancer genomes results in transcriptional repression of tumor suppressors, and treatment of RMS cell lines with the DNA demethylating agent 5-azacytadine results in a differentiation phenotype.[40] Several groups have used a candidate gene approach in RMS tumors to identify methylation changes at the promoters of *FGFR1*, *MYOD1*, and *PAX3*.[41–43] A more global approach using genome-wide DNA methylation arrays was recently reported. The authors reported that both fusion-positive and fusion-negative tumors have distinct methylation profiles, with the fusion-positive subtype showing enriched methylation in genes targeted by the polycomb repressive complex.[44] In addition, they reported promoter methylation of *IRX1*, *DNAJA4*, and *P4HTM* in cell lines and primary tumors that results in the silencing of these genes when compared with normal skeletal muscle. The gene product of *EZH2* is a critical component of the polycomb repressive complex 2 (PRC2), which catalyzes trimethylation of histone H3 lysine 27 and recruits polycomb complexes, DNA methyltransferases, and histone deacetylases (HDACs), resulting in transcriptional repression. Aberrant *EZH2* activity, either as a result of mutation or overexpression, is a common feature of cancer.[45] In addition, *EZH2* expression decreases and the PRC2 dissociates as myogenesis progresses toward mature muscle development.[46] While the critical genes that *EZH2* modulates remain to be elucidated, certainly *EZH2* is highly upregulated in RMS cell lines and tumors.[47] One intriguing study found that the PRC2 in RMS may play a role in preventing the binding of *MYOD1* at muscle-specific genes.[48] *PAX3-FOXO1* itself is a fusion of 2 transcription factors; as such, chromatin immunoprecipitation sequencing analysis using an antibody specific to the fusion junction has been used to interrogate how the oncogene interacts with the genome.[49] Interestingly, the vast majority of *PAX3-FOXO1* binding sites are located more than 4 kb from a transcriptional start site and only infrequently (0.4%) within 1 kb upstream of the transcription initiation site, making it much more likely that the fusion gene exerts its effects through enhancer regions rather than at promoters. Enriched peaks included *PAX3* binding motifs and regions distal to the muscle development genes *MYOD1* (Fig. 3) and *MYF5*. In addition, Cao et al. demonstrated a strong association between chromatin

immunoprecipitation sequencing peaks and genes that are overexpressed in alveolar RMS and confirmed that *ALK*, *FGFR4*, *IGF1R*, and *MYCN* are direct targets of *PAX3-FOXO1*.

IV. PATHOGENESIS AND BIOLOGY OF RMS

A. Fusion-Positive RMS

PAX3-FOXO1 clearly defines a distinct genotype, and given its role as the key prognostic marker in RMS,[50,51] multiple groups have attempted to characterize the effects of the fusion gene on the cell. The translocations create a break within intron 7 of the *PAX* gene and intron 1 of the *FOXO1* gene, resulting in a chimeric transcript that encodes the N-terminal DNA-binding domain of the *PAX* gene with the C-terminal activation domain of the *FOXO1* gene.[52,53] Expression of the fusion gene causes transformation and anchorage-independent growth of fibroblasts.[54] Knockdown of *PAX3-FOXO1* decreased the proliferation rate in the RMS cell line RH30 and the metastatic phenotype observed in the corresponding xenograft model.[55] In addition, conditional simultaneous biallelic PAX3-FOXO1 expression from the *PAX3* locus and homozygous deletion of either *TP53* or *CDKN2A* in Myf6-expressing maturing mouse myofibers leads to alveolar RMS development with 100% penetrance.[56,57]

Expression analysis has been the tool of choice to globally dissect the downstream effects of the presence of the fusion gene. These studies revealed that the oncogene alters the myogenic program of the cell, inducing or repressing a large set of muscle development genes, including *MYOD1*, *MYOG*, and *SIX1*.[59,59] Expression of the fusion protein also massively upregulates several receptor tyrosine kinase molecules important for cell growth, including *FGFR4*,[60] *ALK*,[61] and *MET*,[62] which may provide a feed-forward loop driving proliferation. Universally, these studies have demonstrated a common signature that is associated with a tumor's fusion status and that can be used for diagnosis and prognosis.[51,63]

B. Fusion-Negative RMS as an "RAS-opathy"

As discussed above, the most common causative single nucleotide variant in fusion-negative RMS is an oncogenic mutation in one of the Ras isoforms, namely, *NRAS*, *HRAS*, or *KRAS*. The role of Ras mutation as a prognostic marker in fusion-negative RMS is emerging. In one recent study 75% of high-risk fusion-negative RMS tumors harbored a Ras isoform mutation, in contrast to 45% of intermediate-risk and 0% of low-risk fusion-negative RMS tumors with a Ras isoform mutation.[19] A subset of fusion-negative RMS tumors harbors mutations in the Ras GTPase-activating protein *NF1*, which also potentially leads to aberrant Ras signaling in these tumors. Finally, activating mutations in known Ras effectors such as *BRAF* and *PIK3CA* also have been identified in fusion-negative RMS tumors; however, the signaling pathways downstream of activated Ras that are necessary for tumorigenesis in fusion-negative RMS have yet to be fully characterized. Fusion-negative RMS is unique in that mutations in all 3 of the major Ras isoforms may function as genetic driver mutations.[64] The functional consequences of individual Ras isoform mutations in fusion-negative RMS tumors are not yet known.

The available in vitro and in vivo models of fusion-negative RMS confirm a central role for aberrant Ras activity in fusion-negative RMS tumorigenesis. A knock-in model in which

oncogenic *KRAS* is conditionally expressed in the skeletal muscle of adult mice deficient in *TP53* leads to the development of pleomorphic RMS.[65] In addition, human postnatal skeletal muscle myoblasts engineered to overexpress large T oncoprotein, small t oncoprotein, human telomerase reverse transcriptase, and oncogenic *HRAS* show anchorage-independent growth and form tumors capable of local invasion and distant metastasis when implanted in immunodeficient mice.[66] Most of the established human fusion-negative RMS cell lines currently in use harbor oncogenic mutations in one of the Ras isoforms.[67] Finally, zebrafish embryos injected with oncogenic *KRAS* at the one cell stage develop tumors that histologically resemble fusion-negative RMS.[68] In sum, the available next-generation sequencing and model system data indicate that the major driver of fusion-negative RMS tumorigenesis is oncogenic Ras.

Mutation of *FGFR4* has been identified in approximately 7% of fusion-negative RMSs.[30] Interestingly, *FGFR4* is expressed in myoblasts during normal development and in regenerating muscle following injury, but not in mature skeletal muscle. Based on microarray-based gene expression analysis, this gene has been reported to be the most differentially expressed gene in RMS tumors.[59,63,69] These observations led to the hypothesis that overexpression or mutational activation of this gene may be involved in the tumorigenesis of RMS. Taylor et al. found that suppression of the wild-type *FGFR4* in RMS led to reduced growth and lung metastasis.[16] Mutations in the tyrosine kinase domain were predicted and confirmed to be activating and resulted in increased growth and reduced RMS cell death, and enhanced the ability of RMS cells to metastasize. The investigators found that the mutations lead to the activation of an oncogenic pathway involving *STAT3*.[30] Subsequent studies have shown that stimulation of wild-type *FGFR4* in fusion-positive RMS cell lines leads to activation of the RAS-RAF-MEK-ERK mitogen-activated protein kinase pathway, indicating that RMS tumors expressing activating mutations in *FGFR4* may phenocopy tumors with activating mutations in Ras isoforms.[70]

Insulin-like growth factor (IGF) 2 promotes myoblast proliferation and nutrient uptake. Loss of imprinting at the *IGF2* locus is associated with massive overexpression of IGF2 at the messenger RNA and protein levels in fusion-negative RMS cell lines, and expression of IGF2 is also induced by PAX3-FOXO1.[60] The cellular effects of IGF2 are mediated by the type I IGF receptor (IGF1R), which also is expressed on RMS cells. IGF2 is secreted by RMS cell lines and is able to act as a mitogen in an autocrine manner in this model system.[71] Mouse C2C12 myoblasts expressing IGF2 in combination with PAX3-FOXO1 are transformed in vitro and form undifferentiated tumors in immunodeficient mice.[72]

C. Evidence for a Commonly Disrupted Pathway in Fusion-Positive and Fusion-Negative Tumors

Our genomic characterization of RMS tumors identified some interesting overlap between the genes mutated in fusion-negative tumors and the genes that are under the control of the *PAX3-FOXO1* fusion gene. The results demonstrate that both alveolar RMS and embryonal RMS tumors hijack the common receptor tyrosine kinase/RAS/PIK3CA axis through alternative mechanisms: either mutation or translocation. Up to 93% of RMS tumors have genetic evidence indicating alteration of this axis.[18] With the proliferation of approved

clinical agents that target these pathways, these findings provide a molecular basis for the rapid movement of these agents into clinical trials of RMS.

D. Alteration of Developmental Programs in RMS

Anatomic location of RMS tumors within skeletal muscle and the characteristic expression of muscle markers such as myogenin[73] and *MYOD1*[74] provide evidence that RMS tumors reflect a disordered or corrupted muscle development program. The process of normal myogenic differentiation is driven by sequential expression of myogenic regulatory factors that include the basic helix-loop-helix transcription factors *MYOD1*, *MYF5*, *MYOG*, and *MYF6* (reviewed in refs. 75 and 76). The paired box transcription factors *PAX3* and *PAX7* in turn regulate MYOD1 and MYF5.[77] Forced expression of the *PAX3-FOXO1* fusion alters a myogenic program including upregulation of *MYOD1*, *MYOG*, *SIX1*, and *IGF2* and induces myoblast-like cells.[60] Collectively, these findings indicate that in RMS the oncogenic damage to a muscle precursor cell leads to a persistent or static developmental state that drives both the survival and proliferation signals in the tumor.

V. POTENTIAL NEW THERAPEUTIC OPTIONS

A. Signaling Inhibitors, Including Combination Strategies

Perhaps the most immediate translational clinical opportunity that can be derived from the molecular and genomic study of RMS is that these tumors result from alteration of signaling and growth pathways, many of which can be targeted with small-molecule inhibitors or biological agents (Fig. 4).

The nearly universal alteration of the insulin signaling pathway in RMS tumors has made it an intense focus for novel therapies. Cixutumumab, a human immunoglobulin G monoclonal antibody directed against IGF1R, the receptor for IGF2, showed promising single-agent activity in xenograft models of RMS.[78] Based on these results, 20 pediatric patients with RMS were treated in a phase II clinical trial of cixutumumab as a single agent. In that study, 1 patient had a partial response to anti-IGF1R therapy, 1 patient had prolonged stable disease, and 2 patients had short-term stable disease.[79] The efficacy of cixutumumab in combination with intensive multiagent, interval, compressed cytotoxic chemotherapy was studied in metastatic RMS (www.clinicaltrials.gov identifier NCT01055314). An additional anti-IGF1R antibody, R1507, also showed promising results as a single-agent therapy in patients with RMS: Of 36 patients with RMS treated in the study, 1 patient had a partial response and 6 patients had stable disease.[80] BMS-754807, a small-molecule, ATP-competitive inhibitor of IGF1R, has shown promising activity as a single agent in RMS cell lines in vitro and is currently under investigation in phase I clinical trials for adults with solid tumors.[78]

FGFR4 signaling is altered in both fusion-positive (by overexpression) and fusion-negative (by mutation) RMS, making FGFR4 an attractive candidate for targeted therapy. Recent work demonstrated that the tyrosine kinase inhibitor ponatinib potently inhibited FGFR4 signaling in cell lines expressing either wild-type or mutant FGFR4. This drug also inhibited

growth of RMS cell lines in vitro, induced apoptosis in RMS cell lines, and inhibited tumor growth in xenografts of cell lines expressing mutant FGFR4.[81]

The ALK receptor, a member of the insulin receptor family of receptor tyrosine kinases, activates the signal transducer and activator of transcription 3, PI3K/AKT, and Ras/ERK pathways, and is highly expressed in both fusion-positive and fusion-negative RMS. This high level of protein expression can be the result of ALK gene copy number gain. In addition, *ALK* gene expression is enhanced by PAX3-FOXO1.[61] Genomic gains or amplifications of ALK also occur in pediatric tumor neuroblastoma, as well as non-small-cell lung cancer. Small-molecule inhibitors of ALK (crizotinib, ceritinib) are currently being studied in these tumor types (www.clinicaltrials.gov identifier NCT01742286) and may prove to be beneficial in the treatment of RMS. Additional receptor tyrosine kinases, such as MET, epidermal growth factor receptor, and vascular endothelial growth factor receptor also are overexpressed in RMS compared with normal muscle, and small-molecule inhibitors or antibody-based therapies directed against these receptor tyrosine kinases could also be developed as effective RMS therapies.[82]

RMS likely represents a malignant tumor of myoblast-like cells failing to exit the cell cycle and differentiate. The cell cycle exit and differentiation of myoblasts into myotubes are mediated, in part, by downregulation of cyclin D1 and a decrease in CDK4/CDK6 activity. Genetic amplification of CDK4 and genetic loss of the CDK4/6-specific inhibitor $p16^{Ink4a}$ (CDKN2A locus) frequently occur in RMS, making CDK4 an attractive target in RMS. Treatment of RMS cell lines with the CDK4/6 inhibitor palbociclib (PD-0332991) leads to G^1 arrest and induces the expression of muscle-specific markers. This inhibitor is currently in early phase clinical trials in adults and may represent a therapeutic option for patients with RMS.[83] In addition, as discussed above, the MDM2 locus also is frequently amplified in RMS. An inhibitor of MDM2–TP53 interaction, MI-63, decreases proliferation and increases apoptosis in RMS cell lines expressing wild-type TP53.[84] This compound is currently awaiting phase I testing.

Because Ras isoforms also are commonly mutated in fusion-negative RMS, targeting Ras effectors such as PI3K and BRAF is a logical strategy. Several BRAF (veumurafenib, dabrafenib) and MEK (trametinib) inhibitors have gained US Food and Drug Administration (FDA) approval for the treatment of other Ras-driven cancers, such as metastatic melanoma. Recent work has shown that the small-molecule inhibitors of MEK and PI3K—U0126 and PI103, respectively—have a synthetic lethal interaction in RD, a fusion-negative RMS cell line harboring an activating mutation in *NRAS*.[85] Further studies have shown that growth of RD is dependent on the RAS-RAF-MEK-ERK signaling pathway because an MEK inhibitor, AZD244 (selumetinib), and an ERK2 inhibitor, VX-11e, inhibit proliferation of these cells.[86] Finally, treatment with the MEK inhibitor PD-0352901 leads to differentiation of RD.[87]

Ultimately, single-agent targeted therapy for RMS seems unlikely to provide durable responses. The mechanisms of resistance to therapy are relatively unstudied in RMS; however, some evidence exists that the mechanism likely includes genetic selection of a minor clone that is present at the time of diagnosis.[19] Given the evidence for altered

signaling in RMS through the RAS signaling pathway and the insulin growth signaling pathway, one therapeutic strategy might be to target both the mitogen-activated protein kinase as well as the AKT pathways. This strategy is bolstered by a variety of evidence from adult tumor models showing extensive cross-talk between the 2 pathways. One exciting preclinical study using AZD8055, a mammalian target of rapamycin inhibitor, and AZD6244, an MEK inhibitor, demonstrated significant synergy in both fusion-positive and fusion-negative cell lines both in vitro and in vivo.[88] With the explosion of clinical trials examining similar combinations in adult solid tumors, it seems possible that these results could rapidly inform novel therapies for RMS. Combinations of signaling inhibitors similar to those described above might also be efficacious.

B. Modulating the Epigenome

The convergence point for abnormal signaling in cancer is transcriptional regulation. Therefore, novel mechanisms to target the epigenome might provide new therapeutic modalities for the treatment of RMS. HDACs are critical regulators of gene expression and linked to key oncogenic events in a variety of tumor types, including colon, breast, and lung tumors and leukemia.[89] In preclinical xenograft models of both fusion-positive and fusion-negative RMS, HDAC inhibitors inhibited growth and induced apoptosis.[90] Also, in preclinical models, HDAC inhibition radiosensitized RMS tumors.[91] Finally, in the therapeutically challenging fusion-positive subtype, the combination of HDAC inhibition with the multikinase inhibitor PKC412 released the transcriptional repression of p21, resulting in a synergistic therapeutic combination.[92]

Inhibiting the epigenetic silencing of myogenic genes involved in normal skeletal differentiation by pharmacologic inhibition of EZH2 has restored myogenic differentiation of the embryonal cell line RD in both culture as well as xenograft. Ciarapica et al. [93] used the catalytic inhibitor MC1945 and demonstrated antiproliferative effects as well as increased expression of markers of terminal muscle differentiation compared with placebo. Given the pursuit of EZH2 inhibitors for the treatment of diffuse large B-cell lymphoma, these results provide an exciting new therapeutic avenue in fusion-negative RMS.

C. Directly Targeting the Fusion

A fusion protein that occurs specifically in tumor tissue while being absent in normal tissue theoretically presents an ideal opportunity for a “precision” therapy. In fusion-positive RMS this strategy is further enhanced by the observation that targeted knockdown of *PAX3*[94] or the *PAX3-FOXO1* gene product[95] induces apoptosis and inhibition of proliferation. Unfortunately, unlike the *BCR-ABL* or *EML4-ALK* fusion oncogenes that occur in adult cancers, which involve a targetable tyrosine kinase, the translocations observed in pediatric solid tumors almost uniformly involve transcription factors. While it is generally thought that specific inhibition of the transcriptional machinery is difficult,[96] there has been recent success inhibiting the *EWS-FLI1* translocation in Ewing sarcoma[97] and the *SS18-SSX* translocation in synovial sarcoma.[98] Similar efforts have been completed in *PAX3-FOXO1*-driven RMS, with interesting results. Using a luciferase reporter placed downstream of the promoter of a *PAX3-FOXO1*-induced gene (*TFAP2B*), Martin and colleagues[99] screened a small-molecule library and identified the synthetic retinoid fenretinide as a specific repressor

of *PAX3-FOXO1* at both the messenger RNA and protein levels, which induced apoptosis of RMS cell lines both in vitro and in vivo. A second drug screen using a similar approach identified that fascaplysin, an inhibitor of *CDK4*, worked by abrogating the phosphorylation and subsequent subcellular localization of *PAX3-FOXO1*.[100]

D. Immunotherapy

Antibodies or immune cells engineered against specific antigens expressed by cancer cells have been increasingly used as therapeutic agents either alone or in combination with chemotherapeutic drugs.[101,102] Following the revolutionary success of rituximab targeting CD20 on B cells as an FDA-approved drug for non-Hodgkin lymphoma, a number of monoclonal antibodies have been developed and are being used as approved drugs in the clinic or are currently in various phases of clinical trials for the treatment of leukemias,[103–106] as well as solid tumors including mesothelioma[107] and neuroblastoma.[108,109] Most recently, chimeric antigen receptor–based therapies have shown stunning successes in refractory pediatric cancers.[110] Given their unique expression pattern, including *FGFR4*, RMS tumors present a unique opportunity for immunotherapy.[111] A consortium funded by a StandUp2Cancer and a St. Baldrick's grant is actively pursuing this novel therapeutic modality.

VI. FUTURE PERSPECTIVES

Clear progress has been made in the understanding of the molecular and genetic causes that are the basis of RMS oncogenesis. This groundwork is ushering in a new era in which molecular knowledge will inform risk stratification and clinical decisions in real time. While this goal will be challenging in cases of a relatively rare pediatric tumor, the growing knowledge of the oncogenic and survival mechanisms underlying RMS give clinicians remarkable insights into their patients' disease. Given the mortality associated with high risk and relapsed disease, priority should be given to innovative treatment modalities for these patients. While determining a statistically significant clinical improvement in such a small patient population is daunting, many of the genetic and molecular features of RMS overlap with alterations that occur in more common tumors. Previously established, FDA-approved drugs might provide significant benefit to these patients. In addition, these patients can provide crucial preliminary information for candidate therapies before expanding their use in the intermediate-risk patient cohort. Proper design of these trials should include a tumor biopsy before, during, and upon relapse so that the genomic and proteomic informations can be used to properly link the patient with the appropriate therapy and evaluate how the tumor responds to the treatment. During the same procedure, adequate tissue should be collected so that banking cell lines and in vivo tumor grafts can be generated, allowing the physician and researcher to fully evaluate the tumor's response to the drug. These generated model systems would serve as critical tools to guide the development of novel therapies.

Acknowledgments

The authors thank Berkley Gryder for his help in the preparation of this manuscript. The reviewers are supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply

endorsement by the US government. J.F.S. receives additional grant support from the Sarcoma Research Alliance for Collaboration and the St. Baldrick's Foundation.

ABBREVIATIONS

FDA	US Food and Drug Administration
HDAC	histone deacetylase
IGF	insulin-like growth factor
IGF1R	type I insulin-like growth factor receptor
PI3K	phosphoinositide-3 kinase
PRC2	polycomb repressive complex 2
RMS	rhabdomyosarcoma

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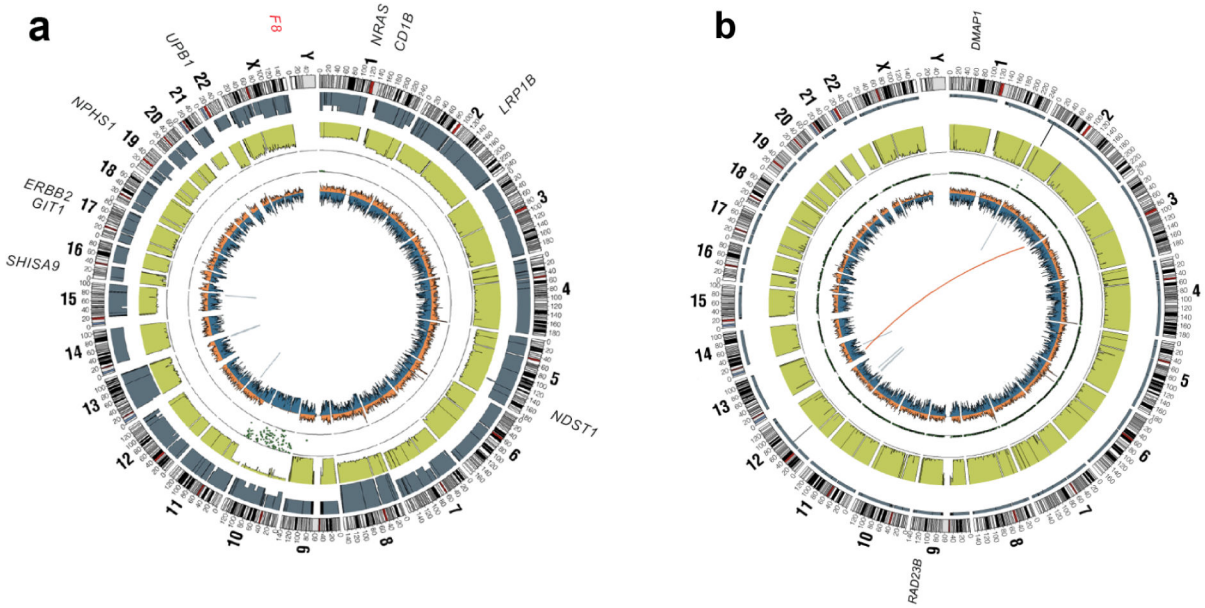

FIG. 1.

Whole-genome sequencing has revealed that rhabdomyosarcoma tumors can be classified into 2 distinct genotypes characterized by the absence (**a**) or presence (**b**) of a *PAX* gene rearrangement. CIRCOS plots from representative tumors are presented (from the outside circle in). Mutated genes: missense mutations (black), non-sense mutations and insertions/deletions (red); genomic location: genome-wide copy number alterations (gray), lesser allele frequency (green), loss of heterozygosity (dotted track), density of heterozygous single nucleotide polymorphisms (SNPs) (orange), homozygous SNPs (blue); intrachromasomal rearrangements (inner circle, gray) and interchromosomal rearrangements (inner circle, red). Adapted from Shern et al.[18]

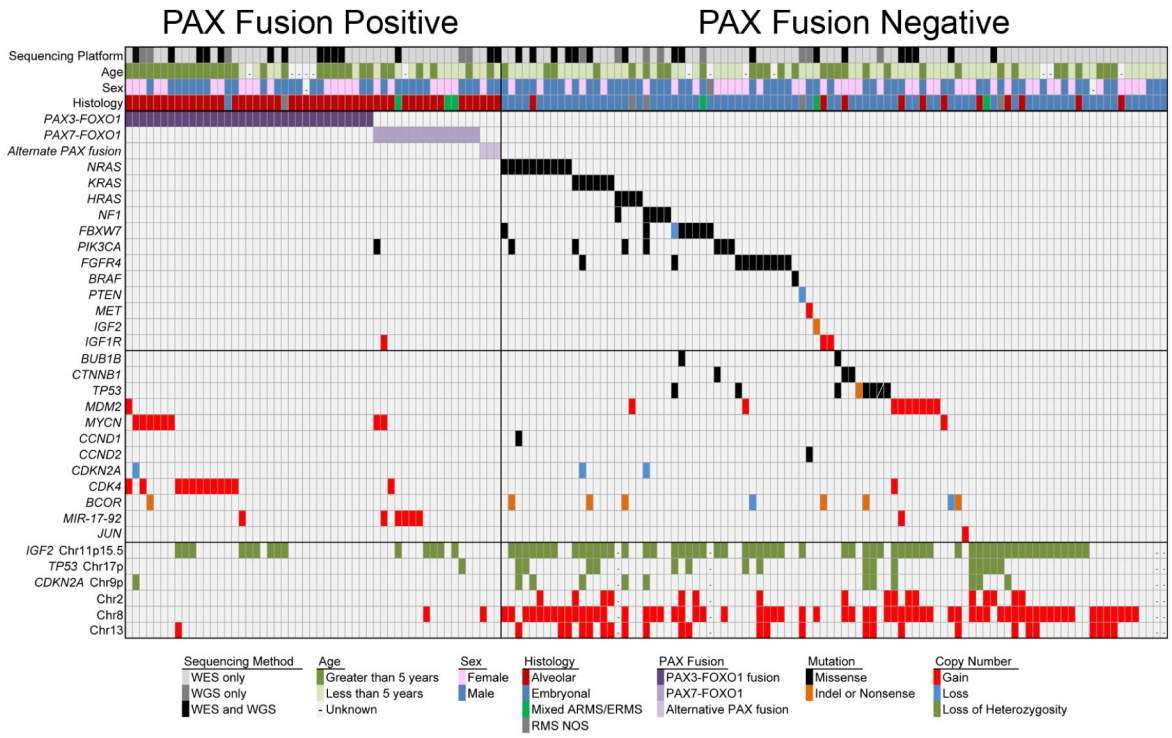

FIG. 2.

A summary of the genomic alterations frequently occurring in primary rhabdomyosarcoma shows 2 distinct genotypes defined by the presence or absence of a *PAX* gene fusion and recurrent mutation of RAS pathway genes in fusion-negative tumors. Adapted from Shern et al.[18]

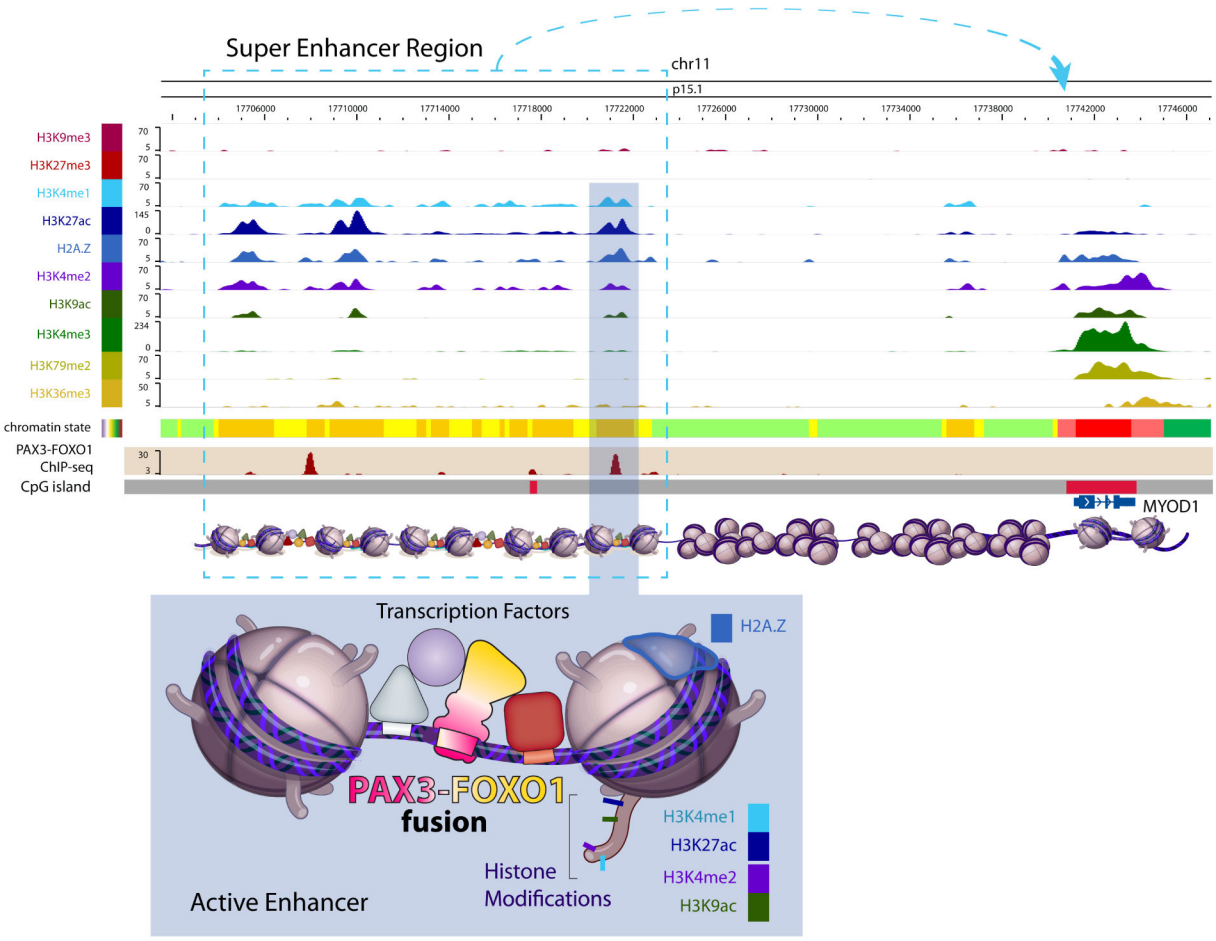

FIG. 3.

The interaction of PAX3-FOXO1 with genomic locations. As determined by chromatin precipitation, the *PAX3-FOXO1* fusion gene is typically associated with enhancer regions of the genome. Presented here are the fusion gene binding sites in the MYOD1 enhancer region (top panel) and its relationship to other epigenetic and transcriptional marks (bottom panel).

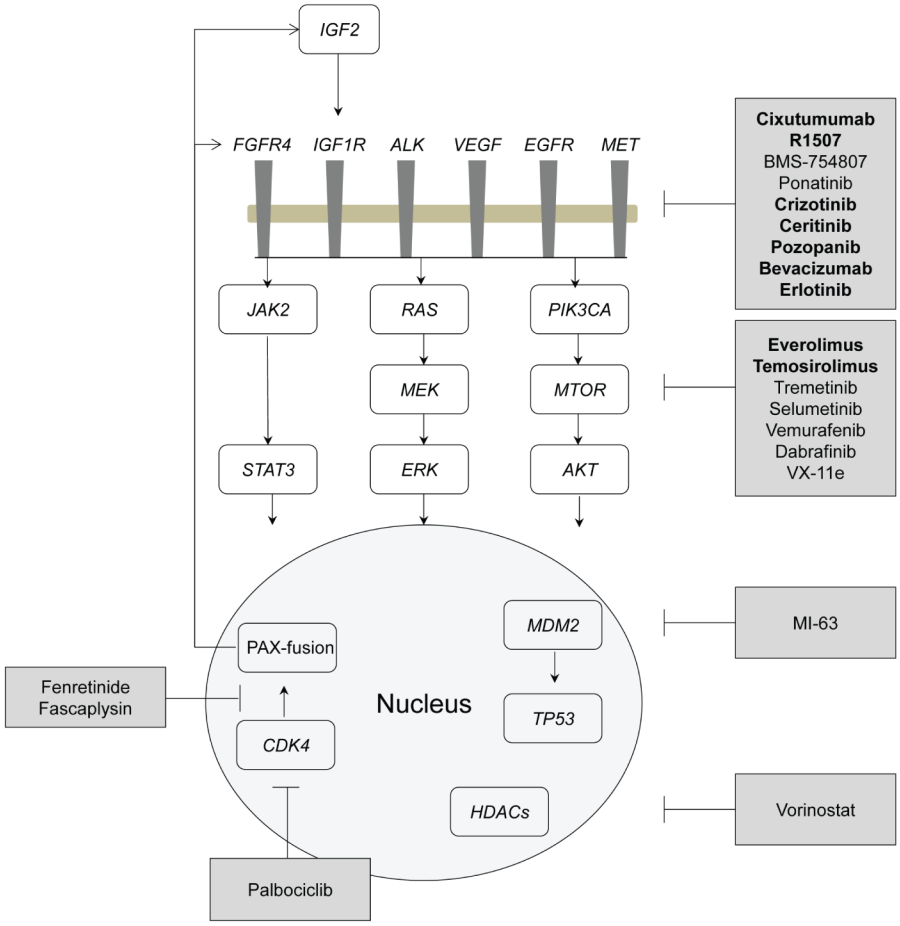

FIG. 4.

Summary of the potential therapeutic options outlined in this review. Adapted from Shern et al.[18]

TABLE 1

Genetic Alterations Commonly Observed in *PAX* Gene Fusion–Positive Rhabdomyosarcoma

Alteration	Type	Chromosomal Locus	Estimated Frequency	References
PAX3-FOXO1	Translocation	(2;13)(q35;q14)	59% of ARMS	17, 24
PAX7-FOXO1	Translocation with amplification	(1;13)(p36;q14)	19% of ARMS	21, 24
PAX3-NCOA1	Translocation	Alveolar	Rare	23
PAX3-INO80D	Translocation	Alveolar	Rare	18
CDK4	Amplification	12q13-14	12%	25
MYCN	Amplification	2p24	20%	25
miR-17-92	Amplification	13q31-32		26
IGF2 locus	Uniparental disomy	11p15.5	29%	18

ARMS, alveolar rhabdomyosarcoma.

TABLE 2

Genetic Alterations Commonly Observed in *PAX* Gene Fusion–Negative Rhabdomyosarcoma

Alteration	Type	Chromosome Locus	Estimated Frequency	References
IGF2	Loss of heterozygosity	11p15.5	65%	18
NRAS	Point mutation	Chr1:115256530 Chr1:115258745	7.5%	18
KRAS	Point mutation	Chr12:25398284 Chr12:25398281	4%	18
HRAS	Point mutation	Chr11:534289	3%	18
NF1	Point mutation	Multiple	3.4%	18
PIK3CA	Point mutation	Chr3:178952084-178952085 Chr3:178936094-178936096	5.4%	18, 29
FBXW7	Point mutation	Chr4:153247287 Chr4:153249456 Chr4:153251905	4.8%	18
FGFR4	Point mutation	Chr5:176522551	9.3%	30
BCOR	Point mutation/indel	Multiple	5.4%	18
CTNNB1	Point mutation	Chr3:41266101 Chr3:41266124	2%	18, 29
MYOD1	Point mutation	p.Leu122Arg	10% of ERMS	20, 31
MDM2	Amplification	12q15	10%	32
Aneuploidy	Chromosome gain	Typically 2, 7, 8, 11, 13	Common	18
	Chromosome loss	Typically 1p, 9, 16	Common	18

ERMS, embryonal rhabdomyosarcoma; indel, insertion/deletion.