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A CALL TO ARMS: TARGETING THE PAX3-FOXO1 GENE IN ALVEOLAR RHABDOMYOSARCOMA*

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Abstract

Introduction: Expression of fusion oncoproteins generated by recurrent chromosomal translocations represents a major tumorigenic mechanism characteristic of multiple cancers, including one-third of all sarcomas. Oncogenic fusion genes provide novel targets for therapeutic intervention. The PAX3-FOXO1 oncoprotein in alveolar rhabdomyosarcoma (ARMS) is presented as a paradigm to examine therapeutic strategies for targeting sarcoma-associated fusion genes.

Areas covered: This review discusses the role of PAX3-FOXO1 in ARMS tumors. In addition to evaluating various approaches to molecularly target PAX3-FOXO1 itself, this review highlights therapeutically attractive downstream genes activated by PAX3-FOXO1.

Expert opinion: Oncogenic fusion proteins represent desirable therapeutic targets because their expression is specific to tumor cells, but these fusions generally characterize rare malignancies. Full development and testing of potential drugs targeted to these fusions are complicated by the small numbers of patients in these disease categories. Although efforts to develop targeted therapies against fusion proteins should continue, molecular targets that are applicable to a broader tumor landscape should be pursued. A shift of the traditional paradigm to view therapeutic intervention as target-specific rather than tumor-specific will help to circumvent the challenges posed by rare tumors and maximize the possibility of developing successful new treatments for patients with these rare translocation-associated sarcomas.

Keywords

fusion gene; PAX3-FOXO1; rhabdomyosarcoma; sarcoma; therapeutic target; translocation

1. Introduction

Sarcomas are a rare, heterogeneous array of mesenchymal tumors that encompass more than 50 subtypes [1–4] Tumors can arise from bone, cartilage, or connective tissues and present virtually anywhere in the body [1,5]. While they account for only 1% of all cancers, sarcomas are more prevalent in children than adults and represent approximately 13% of malignancies affecting patients less than 20 years of age [1, 3].

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Sarcomas have traditionally been divided into two major categories: 1) tumors with nonspecific genetic lesions and complex karyotypes; and 2) tumors with simple genetic alterations and nearly diploid karyotypes [3, 5]. Tumors classified in the second category often arise *de novo* and harbor chromosomal translocations [5]. Although non-random translocations are generally rare in solid tumors, they are associated with about one-third of all sarcomas [6]. Indeed, recurrent translocations have been identified in 19 sarcoma subtypes [2]. The majority of these non-random chromosomal translocations generate chimeric transcription factors, which aberrantly transactivate target gene expression [2, 5]. In Ewing's sarcoma, for example, the common t(11;22)(q24;q12) translocation creates the *EWSR1-FLI1* fusion transcription factor [1]. A different mechanism is demonstrated in dermatofibrosarcoma protuberans (DFSP) where a *COL1A1-PDGFB* fusion constitutively drives PDGFB expression from *COL1A1* regulatory elements [1,2, 5].

Studies of recurrent chromosomal translocations and their associated fusion genes have contributed much to sarcoma research from both basic biology and clinical perspectives. Gene fusions have not only furthered understanding of sarcomagenesis, but have improved diagnosis, as the presence of defined fusion genes can be detected by RT-PCR and FISH approaches [7] Moreover, these specific fusion genes provide additional targets for therapeutic intervention in sarcoma. While advances in targeted therapy have been made in a few categories - for example, PDGFR inhibition by the tyrosine kinase inhibitor imatinib has been demonstrated as an effective therapy for DFSP [1, 8] - useful therapies targeting chimeric transcription factors remain largely undeveloped.

This review will focus on PAX3-FOXO1, the gene product resulting from the chromosomal translocation t(2;13)(q35;q14), in alveolar rhabdomyosarcoma (ARMS). PAX3-FOXO1 in ARMS is highlighted as an excellent paradigm to target sarcoma-associated fusion genes based on the following: 1) development of skeletal muscle, which is the lineage related to ARMS tumors, has been extensively described [9]; 2) wild-type *PAX3, PAX7* [10–14], and *FOXO1* [15–17] as well as resulting chimeric products [18–21] have been well characterized; and 3) model systems—comprising both cell culture [22–26] and whole animal, including a conditional knock-in mouse model of PAX3-FOXO1-induced ARMS [27–30]—have been developed. Through examination of PAX3-FOXO1, this review will discuss a conceptual framework of therapeutic strategies that applies not only to this ARMS-specific gene fusion, but also to oncogenic transcription factor chimeras of other sarcoma subtypes.

2. Rhabdomyosarcoma (RMS) Family of Tumors

RMS is a heterogeneous family of pediatric soft tissue tumors associated with the skeletal muscle lineage [31]. Although rare in adults, RMS is the most common pediatric soft tissue sarcoma, accounting for approximately 50% of all soft tissue sarcomas in children and adolescents [32, 33]. With an annual incidence of 4.5 cases per million children in the United States, which corresponds to roughly 350 new cases per year, RMS represents about 3–4% of all childhood malignancies [33–35].

Alveolar rhabdomyosarcoma (ARMS) and embryonal rhabdomyosarcoma (ERMS) constitute the two major histopathologic subtypes of RMS. These two variants are not only

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histologically distinguishable, they are also associated with clinically distinct phenotypes [32, 36]. ARMS accounts for 20–30% of RMS, affects children as well as adolescents and young adults, and tends to occur in the extremities and trunk [32, 33, 36]. In contrast, ERMS represents 70–80% of all RMS cases [32, 33]. Typically presenting in patients less than 10 years of age, ERMS predominantly occurs in the head and neck and genitourinary tract [36]. ARMS is clinically more aggressive than ERMS and is associated with an unfavorable prognosis, which is partially attributable to its propensity for early dissemination, poor response to therapy, and frequent relapses following therapy [21, 31, 32, 37, 38]. The 5-year overall survival for ARMS is ~50% compared to ~75% for ERMS [34]

2.1 Molecular Genetics of ARMS

The considerable clinical and pathologic dissimilarities between ARMS and ERMS reflect genetic differences between these RMS subtypes. In contrast to 11p15.5 allelic loss and point mutations that occur frequently in ERMS [32, 33, 39–44], recurrent chromosomal translocations characterize 70–80% of ARMS tumors [33, 45]. Two specific translocations are unique to ARMS tumors: the majority of ARMS cases harbor the common translocation, t(2;13)(q35;q14), whereas a smaller subset of ARMS harbor a variant translocation, t(1; 13)(p36;q14) [7, 33, 45]. In these translocations, *PAX3* is the gene rearranged on chromosome 2 [46], and *PAX7* is the gene rearranged on chromosome 1 [19] (Figure 1). *PAX3* and *PAX7* encode highly homologous members of the paired box family of transcription factors. Structurally, both proteins contain N-terminal DNA binding domains, which are comprised of paired box and homeobox motifs, and C-terminal transcriptional activation domains [11, 32]. The gene located at the chromosome 13 locus in these translocations is *FOXO1,* encoding a member of the O subfamily of forkhead box transcription factors [18, 19, 32]. FOXO1 contains a forkhead DNA binding domain at its N-terminus and a transcriptional activation domain at its C-terminus [6].

The 2;13 and 1;13 translocations break within the seventh intron of *PAX3* or *PAX7* and within the first intron of *FOXO1* [32]. Chimeric genes are thereby generated and encode chimeric proteins consisting of the PAX3 or PAX7 N-terminal DNA binding domain fused to the FOXO1 C-terminal transactivation domain [20, 32] (Figure 1). PAX3/PAX7 and FOXO1 coding sequences are fused in-frame, creating functional—albeit aberrant—transcription factors. PAX-FOXO1 fusion proteins are discussed in greater detail below.

Molecular pathology studies of the chimeric products reveal that ~60% of ARMS tumors are PAX3-FOXO1-positive, ~20% are PAX7-FOXO1-positive, and ~20% are fusion-negative [45,47] These studies thus confirm that there is a subset of histologically defined ARMS tumors that are negative for the hallmark translocations generating PAX3-FOXO1 or PAX7-FOXO1 [33]. In rare cases, alternative translocations, such as t(2;2)(p23;q35) and t(2;8)(q35;q13), result in fusion of *PAX3* to nuclear receptor coactivator genes *NCOA1* and *NCOA2,* respectively [48]. Most cases in this PAX3-FOXO1 and PAX7-FOXO1-negative subset show no detectable rearrangements involving PAX3, PAX7, or FOXO1, providing evidence for *bona fide* fusion-negative ARMS cases [49]. Interestingly, fusion-negative ARMS demonstrates genetic changes characteristic of ERMS, which is consistent with the similar expression patterns [50] and clinical outcomes [51] of fusion-negative ARMS and ERMS cases.

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2.2 PAX-FOXO1 Oncogenicity

The PAX-FOXO1 fusion products have altered expression, subcellular localization, and function, compared to wild-type PAX3, PAX7, or FOXO1. Both PAX-FOXO1 fusion proteins are expressed at higher levels than their wild-type PAX counterparts; PAX7-FOXO1 overexpression results from gene amplification while PAX3-FOXO1 overexpression occurs via copy number-independent enhanced transcription [52]. In contrast to the wild-type FOXO1 protein that can shuttle between the nucleus and cytoplasm, the PAX3- or PAX7-FOXO1 protein is localized exclusively in the nucleus. Finally, these fusion proteins activate transcription of target genes 10–100 fold more potently than wild-type PAX3 and PAX7 [32, 53, 54]

Numerous studies have demonstrated the oncogenic capacity of the PAX3/PAX7-FOXO1 fusion protein. In chicken embryo fibroblasts and murine NIH 3T3 fibroblasts, ectopic expression of PAX3-FOXO1, but not wild-type PAX3, resulted in transformation as evidenced by focus formation and anchorage-independent growth in soft agar [55–57] Based on these early studies, PAX3-FOXO1 appears to function as a dominant-acting oncogene [36]. This fusion likely contributes to tumorigenesis through several mechanisms [58]. The finding that an engineered PAX3-KRAB repressor suppressed the oncogenicity of Rh30 ARMS cells *in vitro* and *in vivo,* supports the hypothesis that PAX3-FOXO1's aberrant transcriptional activity lies at the heart of its oncogenic potential [59].

Despite early reports demonstrating the transformative capacity of the PAX3-FOXO1 fusion [55–57], additional studies revealed that PAX3-FOXO1 is generally not sufficient for complete oncogenic transformation [33, 58, 60]. Ectopic expression of PAX3-FOXO1 alone failed to result in transformation of human myoblasts or murine mesenchymal stem cells [25, 61]. In fact, high expression levels of PAX3-FOXO1, comparable to endogenous fusion expression levels in human ARMS tumor cells, were found to be anti-proliferative in immortalized murine cell lines[62].

In collaboration with added genetic lesions, PAX3-FOXO1 expression is capable of transforming human and murine cells to recapitulate ARMS tumors [33, 58]. For example, immortalized human myoblasts expressing PAX3-FOXO1 were transformed upon introduction of MYCN [61]. Similarly, p53 inactivation was required in PAX3-FOXO1-expressing murine mesenchymal stem cells to elicit ARMS-like tumor formation when injected into immunocompromised mice [25]. Human skeletal muscle myoblasts stably expressing PAX3-FOXO1 can produce ARMS-like tumors after addition of *TERT* and *MYCN* and loss of *CDKN2A* [26].In studies of a mouse model using a conditional *PAX3-FOXO1* knock-in allele, ARMS formed at low frequency, but addition of conditional *Trp53* or *Cdkn2a* inactivation increased ARMS tumor incidence, providing further evidence that the PAX3-FOXO1 fusion requires accompanying genetic lesions for ARMS pathogenesis [27]

Although PAX3-FOXO1 and PAX7-FOXO1 are virtually indistinguishable in structure [31],PAX3-FOXO1 expression portends an especially unfavorable outcome relative to PAX7-FOXO1 [63]. In recent analyses of a cohort of fusion-positive RMS, PAX7-FOXO1 fusion status demonstrated a statistically significant association with improved overall survival ($p=0.0012$) [63]. The poorer prognosis associated with PAX3-FOXO1-positive tumors coupled

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with the ARMS- specific nature of PAX3-FOXO1 expression make this oncogenic chimera a very appealing therapeutic target. It is worth noting that, to date, most functional studies have concentrated on PAX3-FOXO1, though many findings can be extended in conception to include PAX7-FOXO1. This review will focus on molecular therapeutic strategies to abrogate oncogenic activity driven by the PAX3-FOXO1 fusion in ARMS.

3. Targeting PAX3-FOXO1

3.1 Regulating PAX3-FOXO1 Expression

3.1.1 RNA Interference and Antisense Technologies—While PAX3-FOXO1 expression is typically not sufficient for full oncogenic transformation [33, 58, 60], the fusion protein plays a necessary and fundamental role in ARMS tumorigenesis [64]. Indeed, reduced cellular proliferation, decreased motility and invasion, and increased myogenic differentiation were observed upon PAX3-FOXO1 depletion [64] These phenotypic effects can be attributed to PAX3-FOXO1, as depletion of the oncogenic chimera was achieved by siRNA specifically targeting the PAX3-FOXO1 fusion [64] Furthermore, in another study that selectively decreased expression of PAX3-FOXO1 using shRNA directed against the PAX3-FOXO1 fusion point, PAX3-FOXO1-expressing human myoblast-derived tumor cells and ARMS cells displayed significantly reduced proliferation rates and transformation capabilities coupled with elevated myogenic differentiation relative to cells transduced with control shRNA [61].

PAX3-FOXO1 may also be required for cell survival, though findings are not as conclusive as those described above. Antisense oligonucleotide- or siRNA-mediated depletion of PAX3-FOXO1 induced apoptosis, suggesting that the fusion protein is essential in cell viability [65, 66]. The caveat is that these approaches [65, 66] were directed toward 5’ PAX3 sequences and thereby targeted wild-type PAX3 as well as the fusion, thus compromising a definitive interpretation of PAX3-FOXO1 as anti-apoptotic.

The anti-tumor effects of PAX3-FOXO1 depletion provide proof of principle for therapeutic strategies designed to abrogate PAX3-FOXO1 expression. Although additional technological advances are required, siRNA/shRNA approaches targeting the oncogenic PAX3-FOXO1 fusion may become a viable method for therapy. Despite existing limitations that impede full clinical translation of antisense therapeutics [67], antisense oligonucleotide-mediated depletion of PAX3-FOXO1 is a potential therapeutic alternative. In a related study, antisense oligodeoxynucleotide treatment against *EWSR1-FLI1* in Ewing’s sarcoma induced tumor regression [68]. Moreover, *EWSR1-FLI1-targeting* antisense oligonucleotides loaded onto nanosphere-chitosan resulted in efficient and tumor-specific delivery of the antisense oligonucleotides [69].

3.1.2 Other Translational or Post-Translational Mechanisms—Preliminary studies of RMS cell lines *in vitro* identified camptothecin as a selective chemotherapeutic agent in ARMS [70]. Camptothecin is a topoisomerase I inhibitor, and its derivatives topotecan and irinotecan have both been evaluated for their utility in RMS in Phase II clinical trials, though neither significantly improved survival [32]. Interestingly, the sensitivity of ARMS cells to camptothecin appeared to depend not on topoisomerase I, but on the transcriptional

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activity of PAX3-FOXO1, as ectopic expression of PAX3-FOXO1 in ERMS cells increases sensitivity to campothecin [70]. Further studies revealed that camptothecin reduces PAX3-FOXO1 transactivation by decreasing its protein expression. Camptothecin-mediated downregulation of fusion protein levels was not attributable to AKT dephosphorylation, p53 function, or reduced PAX3-FOXO1 mRNA expression. Collectively, these data suggest that camptothecin may enhance degradation of the PAX3-FOXO1 fusion protein, and camptothecin is thus postulated to modulate PAX3-FOXO1's ubiquitination status [70]. Ubiquitylation of PAX3-FOXO1 has been demonstrated previously [71], and additional studies of camptothecin may provide a paradigm for therapeutic strategies to stimulate proteasomal degradation of oncogenic fusion protein.

3.2 Regulating the Phosphorylation Status of PAX3-FOXO1

3.2.1 The C-Terminal FOXO1 Portion—As a member of the FOXO transcription factor family, wild-type FOXO1 is regulated by a variety of posttranslational modifications, including deacetylation, ubiquitination, and phosphorylation [15]. The FOXO1 protein shuttles between the nucleus and cytoplasm, with its subcellular localization regulated by the canonical PI3K/AKT signaling pathway [15, 16, 31] (Figure 2). Phosphorylation of FOXO1 confers cytoplasmic sequestration, and dephosphorylation allows nuclear translocation [16] (Figure 2). While multiple serine/threonine kinases, such as members of the AGC protein kinase family [72], CDK1 [73], CDK2, CK1, and DYRK1 have been reported to phosphorylate FOXO1 at various sites, AKT is regarded as the primary kinase involved in phosphorylation-dependent modulation of FOXO1 subcellular localization and consequent transcriptional activity [15–17] FOXO1 harbors three evolutionarily conserved AKT phosphorylation sites located at threonine 24, serine 256, and serine 319 [17] Upon activation, AKT translocates to the nucleus and directly phosphorylates FOXO1 [74–76]. AKT-directed phosphorylation at these residues appears to have no direct consequences on FOXO1 function, but rather facilitates 14–3-3 protein docking and binding, leading to cytoplasmic accumulation of this complex [17] (Figure 2). Thus, phosphorylation by AKT indirectly inactivates FOXO1 transcriptional function.

PAX3-FOXO1 retains two of the three consensus AKT phosphorylation residues found in wild-type FOXO1 [32]. Using a FOXO1 mutant in which threonine 24 was replaced by alanine but serines 256 and 319 were unaltered, studies demonstrated that the presence of these two AKT phosphorylation sites matching those preserved in the PAX3-FOXO1 chimera are sufficient for AKT-mediated cytoplasmic sequestration and inhibition of FOXO1 transcriptional activity [77] A promising prediction was derived: if serine 256 and serine 319 in the FOXO1 portion of PAX3- FOXO1 were phosphorylated by AKT, then PAX3-FOXO1 should be relocalized to the cytoplasm, thereby quelling its ability to aberrantly transactivate target gene expression. In HEK293T and NIH 3T3 cells, transfection of PAX3-FOXO1 or a form with the two serines mutated, with or without constitutively active AKT, showed no effect on PAX3-FOXO1 nuclear localization or transcriptional activity [77]. Possible explanations are that PAX3-FOXO1 adopts a conformation that precludes phosphorylation of relevant FOXO1 residues or that nuclear localization is controlled by N-terminal PAX3 domains.

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A recent study using murine cell lines derived from conditional *PAX3-FOXO1* knock-in mice and cultured in low serum provided evidence that PAX3-FOXO1 can be phosphorylated by hyperactivated AKT in this setting [78]. Phosphorylation rendered PAX3-FOXO1 transcriptionally inactive but induced no change in nuclear localization of the fusion protein [78]. The persistent nuclear presence of PAX3-FOXO1 suggests that, even if PAX3-FOXO1 undergoes phosphorylation, it is not conducive to 14–3-3 protein binding and resultant cytoplasmic sequestration.

While interesting, there is no available data to indicate that such AKT hyperactivation—and resulting PAX3-FOXO1 inactivation—can be induced in human ARMS tumors. IGF2 is overexpressed in human ARMS cells [31], and high phospho-AKT levels were observed in human ARMS tumors and cell lines, indicating the presence of endogenous AKT activation [79]. Thus, human ARMS tumors retain PAX3-FOXO1 transcriptional activity despite the presence of activated AKT. Regardless of the mechanism conferring fusion protein resistance to AKT-mediated regulation in human ARMS, therapeutic approaches would likely be ineffective if they aimed at controlling PAX3-FOXO1 subcellular localization or transcriptional activity by modulating phosphorylation of the FOXO1 portion.

3.2.2 The N-Terminal PAX3 Portion—Compared to phosphorylation of FOXO1, kinases, residues, and functional consequences associated with PAX3 phosphorylation are poorly understood. Additionally, because wild-type PAX3 is exclusively nuclear [80, 81], phosphorylation status of the PAX3 region of PAX3-FOXO1 cannot be exploited using the same conceptual subcellular relocalization framework that applied to the fusion's FOXO1 portion. *In vitro* and *in vivo* studies of murine primary myoblasts revealed serine 201, serine 205, and serine 209 as the only sites of phosphorylation in wild-type PAX3, and all three serine residues are retained in the oncogenic PAX3-FOXO1 fusion [82]. CK2 and GSK3P have been identified as the kinases that phosphorylate wild-type PAX3 and PAX3- FOXO1 at serines 205 and 201, respectively [82, 83]. Recently, CK2 was also found to be responsible for serine 209 phosphorylation [84]

Wild-type PAX3 and PAX3-FOXO1 demonstrate distinct patterns of phosphorylation throughout early myogenic differentiation, leading to proposal of separate models for wild-type PAX3 versus PAX3-FOXO1 fusion phosphorylation [82]. It appears that wild-type PAX3 undergoes GSK3β-mediated phosphorylation at serine 201 only after phosphorylation at serine 205 by CK2. Serine 201 phosphorylation subsequently promotes serine 205 dephosphorylation, as evidenced by the absence of PAX3 species exhibiting simultaneous phosphorylation at serines 201 and 205. Phosphorylation at serine 201 persists as phosphorylation at serine 209 becomes detectable [82].

Experiments in mouse primary myoblasts and human ARMS cell lines indicate that the oncogenic PAX3-FOXO1 fusion is phosphorylated by CK2 at serine 205 followed by GSK3β-driven phosphorylation at serine 201. In contrast to wild-type PAX3, however, these coincident phosphorylations are then maintained throughout early differentiation, and phosphorylation at serine 209 is never detected in PAX3-FOXO1 [82]. The precise functional consequences of each of these phosphorylation events have not yet been elucidated, though the notion that an altered phosphorylation status of the 5' PAX3 portion of PAX3-FOXO1

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relative to wild-type PAX3 contributes to ARMS is provocative. Mutational analyses of PAX3-FOXO1 engineered to mimic wild-type PAX3 phosphorylation patterns and vice versa as well as identification and characterization of additional putative phosphorylation sites, kinases, and phosphatases involved in PAX3 phosphorylation are needed and may inform a new avenue of therapeutic development for ARMS tumors.

Studies of a small-molecule inhibitor have already provided evidence that inhibiting phosphorylation of the PAX3 region of PAX3-FOXO1 can have anti-tumorigenic effects in ARMS cell lines and xenograft models [85]. PKC412, an inhibitor of multiple kinases such as PKC, FGFR, AKT, FLT3, CDK1, and c-Kit, is a staurosporine derivative that suppresses ARMS proliferation and induces caspase 3-dependent apoptosis *in vitro* and reduced proliferation, increased apoptosis, and inhibited tumor growth *in vivo* [85]. PKC412 treatment decreases DNA binding of PAX3-FOXO1, thereby abrogating its transcriptional activity in a phosphorylation-dependent mechanism without affecting its nuclear localization [85]. Unlike the previously described studies identifying three PAX3 serine residues as phosphorylation sites [82], investigators here detected six potential phosphorylation residues at serines 187, 193, 197, 201, 205, and 209. It should be noted that simultaneous mutation of all six serines to phosphorylation-mimicking aspartate residues was required to overcome PKC412 inhibition but was not sufficient to rescue complete transcriptional activity of PAX3-FOXO1 [85], suggesting that PKC412 inhibits fusion protein transactivation potential by mechanisms beyond these six phosphorylation events. Despite concerns of potential toxicity from off-target effects, this study demonstrates that small- molecule-mediated modulation of PAX3-FOXO1 post-transcriptional modifications, namely phosphorylation, is a promising approach for ARMS treatment.

3.3 Recognizing PAX3-FOXO1 as a Tumor Antigen

In pediatric sarcomas, translocation fusion products have long been regarded as potential tumor antigens [86, 87] In particular, it was hypothesized that tumor-specific peptides spanning the translocation breakpoint are proteolytically processed, bound to major histocompatibility complex (MHC) class I molecules, and presented on the surface of the tumor cell [86, 87] Displayed peptides could thereby target tumor cells for recognition and killing by $CD8^+$ cytotoxic T cell lymphocytes (CTL) [86, 87] In a pilot study, apheresis fractions of monocytes and dendritic cells were pulsed with synthetic peptides comprising the PAX3-FOXO1 fusion breakpoint region sequence, and peptide pulsed vaccines were administered with interleukin-2 (IL-2) to ARMS patients [88]. Vaccination, however, failed to affect clinical outcome [88].

In a subsequent study of immunotherapy in ARMS, dendritic cells were pulsed with a specific PAX3-FOXO1 fusion protein breakpoint peptide identified to bind HLA-B7 MHC class I molecules [89]. These dendritic cells were then used to generate a lymphocyte-derived human CTL line capable of lysing PAX3-FOXO1 and HLA-B7-expressing ARMS tumor cells, but not PAX3-FOXO1 fusion-negative ERMS cells [89]. Although this neoantigen is specific for only tumor cells expressing the oncogenic fusion protein and thus allows for highly targeted immunotherapy, HLA-B7 is expressed in less than 25% of the population, suggesting that the majority of ARMS patients is unlikely to benefit from this therapy [89].

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Other MHC class I molecules, including HLA-A1, HLA-A2, and HLA-A3, have been evaluated, but none were found to present neoantigens corresponding to the PAX3-FOXO1 fusion breakpoint region [90].

The most promising data comes from a more recent pilot study of consolidative immunotherapy [91]. ARMS patients in remission following multimodal therapy were vaccinated with dendritic cells pulsed with PAX3-FOXO1 fusion protein breakpoint peptides in combination with autologous lymphocyte infusions with or without IL-2. This consolidation therapy regimen was well tolerated, and ARMS patients who received immunotherapy demonstrated significantly improved survival compared to those who did not [91]. Results should be interpreted with caution, however, as patients with rapidly progressive disease were excluded from immunotherapy in this study. Therefore, it is likely that the observed increase in survival rate is at least partially attributable to patient selection [91]. Nevertheless, immunotherapy represents a plausible strategy to antagonize the PAX3-FOXO1 oncoprotein. Investigations to optimize cancer immunotherapeutic efficacy, including alternative methods for enhancement of antigen immunogenicity and induction of dendritic cell maturation, are underway [91].

4. Targeting Downstream Factors of PAX3-FOXO1

Given the uniqueness of PAX3-FOXO1 to ARMS tumors, the oncogenic fusion itself is a very desirable therapeutic target. Nearly two decades after its initial characterization [18, 46], however, the chimeric transcription factor remains a difficult pharmacological target. Thus, it has become necessary to conceive of alternative treatment approaches.

Much progress has been made in recent years to generate a gene expression profile of ARMS that is distinct from that of ERMS. Multiple downstream genes activated by PAX3-FOXO1 have been identified from this ARMS expression profile, and provide another important source of potential targets for therapeutic intervention [4, 30, 66, 92–100]. While this discussion is not all-inclusive, the presentation below and in Table 1 highlights the most therapeutically promising genes downstream of the PAX3-FOXO1 oncoprotein. Our selections are strongly based not only on molecular targets that promote ARMS tumorigenesis and metastasis, but also on those that contribute to tumor development, maintenance, and progression in other cancer categories.

4.1 FGFR4

Fibroblast growth factor receptor 4 (*FGFR4*) encodes a member of the FGFR family of receptor tyrosine kinases (RTK) that is necessary during normal myogenic differentiation and injury-induced muscle regeneration, but not in mature, differentiated skeletal muscle [101–104]. In RMS, *FGFR4* was identified to be overexpressed at the mRNA and protein levels [105–108]. Analysis of primary RMS tumors revealed a strong correlation of high FGFR4 expression with PAX3-FOXO1-positive ARMS [37, 107], advanced clinical stage, and lower overall survival [109]. In functional studies using Rh30 cells, FGFR4 depletion mediated by inducible shRNA targeting FGFR4 inhibited proliferation *in vitro* and reduced proliferation and lung metastasis *in vivo* [109], further suggesting that FGFR4 is oncogenic in RMS. In addition, activating mutations in the tyrosine kinase domain of *FGFR4* were found in 7.5%

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of RMS tumors (mostly ERMS) [95], and transduction of murine RMS772 cells with two of these FGFR4 mutants resulted in elevated proliferation, invasion, and metastatic capacity relative to wild-type FGFR4-transduced cells *in vitro* and *in vivo* [109]. Recent findings in primary mouse myoblasts demonstrated that ectopic expression of a constitutively active FGFR4 mutant, but not wild-type FGFR4, is sufficient to contribute to ARMS tumorigenesis [110].

Subsequent work demonstrated that *FGFR4* is a direct transcriptional target of PAX3-FOXO1 [111], elucidating a second, mutation-independent mechanism for FGFR4 activation. This additional mechanism of overexpression is consistent with the observation that FGFR4 expression is higher in ARMS tumors than in PAX3-FOXO1-lacking ERMS tumors [109112]. Using ChlP-seq, two PAX3-FOXO1 binding sites were identified downstream of *FGFR4,* and functional examination revealed that one of the binding sites is a *bona fide* PAX3-FOXO1-dependent enhancer [111]. These data solidified the role of FGFR4 as an important oncoprotein in RMS—a mutant protein in a subset of ERMS and an overexpressed wild-type protein in most fusion-positive ARMS.

As a kinase, FGFR4 is inherently more amenable to pharmacologic inhibition than the PAX3-FOXO1 transcription factor. In a very promising finding *in vitro,* FGFR4 mutant-expressing RMS772 cells exhibited enhanced sensitivity to FGFR inhibitor PD173074, as apoptotic cell death was higher in RMS772 cells transduced with FGFR4 mutants compared to those expressing wild-type FGFR4 [109]. PD173074 also attenuated cell proliferation of ARMS and ERMS cell lines overexpressing wild-type FGFR; therefore, FGFR mutations are not required for pharmacologic efficacy of PD173074 *in vitro* [112]. In the *in vivo* setting, however, the small-molecule inhibitor was found to have a narrow therapeutic window and high toxicity, eliminating PD173074 as a viable option in ARMS therapy [112]. Nonetheless, FGFR4 is clearly a very attractive candidate for targeted therapy in RMS and especially in ARMS.

Given that resources are limited, it is most prudent and efficient to develop targeted therapies with applicability to multiple cancer categories. Evidence not only indicated that ARMS tumors are likely addicted to the FGFR4 oncogene [109, 112–115], but FGFR4 has also been proposed as an oncogene in other tumor types, such as cancers of the liver, pituitary, lung, breast, and prostate [116–122]. Thus, FGFR4 represents a prime target for pharmacologic intervention, particularly in the adjuvant setting. Other small-molecule inhibitors targeting FGFRs, including AZD4547 and AZ12908010, have already been developed and are undergoing preclinical and clinical evaluation [123, 124] Moreover, neutralizing or high-affinity monoclonal antibodies against FGFR4 have been generated, and strategies to target FGF19 —the ligand of FGFR4—are under investigation [125–127].

4.2 MET

Like *FGFR4, MET* encodes a RTK proto-oncogene. Upon activation by hepatocyte growth factor (HGF, also referred to as scatter factor) binding, MET promotes cellular proliferation, motility, invasion, and survival [128, 129]. Collectively, the cellular responses evoked by MET have been referred to as “invasive growth,” [130–132] a program that, if dysregulated, can have profound tumorigenic and metastatic consequences.

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Amplification of *MET,* gain-of-function mutations, and transcriptional upregulation are mechanisms leading to MET overexpression and/or activation, which has been reported in a myriad of human primary tumors [128]. Amplification-driven MET overexpression and constitutive kinase activation have been observed in medulloblastomas, esophageal and gastric carcinomas, and colorectal cancer [133–138]. Additionally, MET amplification was found in non-small cell lung cancers with acquired resistance to erlotinib or gefitinib, two epidermal growth factor receptor inhibitors [139, 140]. Activating mutations in *MET* were detected in pediatric hepatocellular carcinoma, head and neck squamous cell carcinoma, papillary renal cancer, gastric cancer, and melanoma [128, 131, 141]. Remarkably, nearly all carcinoma types, such as breast, colorectal, hepatocellular, oral squamous cell, ovarian, pancreatic, prostatic, renal cell, and thyroid exhibit elevated MET expression resulting from transcriptional upregulation [128, 132, 141]. In glioblastoma, osteosarcoma, breast carcinoma, and RMS [128], expression of HGF has been proposed to aberrantly activate MET through an autocrine loop [33, 128, 142].

Expression of MET in RMS is associated with migration, invasion, and metastasis [142–144] Thus, it is not surprising that high MET expression levels correlated with advanced stage, worse outcome, and ARMS histology, specifically PAX3-FOXO1 expression [145, 146]. Although other studies found MET expressed in ERMS as well [147, 148], high MET expression is consistently observed in ARMS [143, 145, 146, 149, 150], whereas its expression levels are more variable in ERMS [147] These findings are in agreement with reports that *MET* is a PAX3-FOXO1 target gene that plays an essential role in mediating the fusion protein's oncogenicity [5, 147, 149]. Importantly, both ARMS and ERMS tumors demonstrated oncogene addiction to MET, as MET depletion abrogated proliferation, invasiveness, survival, and anchorage-independent growth *in vitro* and arrested tumor growth *in vivo* [147] Therefore, both RMS subtypes—in addition to the multitude of cancer types described above—could potentially benefit from MET-directed therapies.

Many therapeutic agents targeting MET have been developed (Table 1) and are at stages ranging from preclinical evaluation to Phase II clinical trials [128, 151]. The sheer variety of compounds and strategies designed to antagonize MET holds much promise: HGF antagonists to block interaction of MET with its ligand, HGF and MET neutralizing antibodies to interfere with HGF-MET binding and to downregulate MET, MET decoys to sequester HGF and obstruct receptor dimerization, and small-molecule inhibitors to impair catalytic activity [128]. MET inhibitors have been discussed comprehensively in a recent review [128]. As clinical trials of MET inhibitors progress in other tumor types, evaluation of their safety and efficacy in RMS is clearly warranted and anticipated.

4.3 MYCN

Unlike the RTKs *FGFR4* and *MET, MYCN* belongs to a transcription factor family of proto-oncogenes that includes *MYC* and *MYCL* [152, 153]. MYCN is a basic helix-loop-helix/leucine zipper transcription factor that is expressed predominantly in neuronal tissues during embryogenesis [153, 154] Following heterodimerization with MAX, MYCN activates transcription of target genes, such as *TERT, ODC, MDM2,* and *IGF1R* [155]. Recent reports

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that the related MYC protein is a global gene expression amplifier [156, 157], however, may foreshadow a comparable, more universal model of gene expression regulation for MYCN.

Normal MYCN expression is virtually undetectable in mature, post-embryonic tissues [153, 154, 158]. Aberrant expression of MYCN as a result of *MYCN* amplification, however, has been detected in several malignancies, including neuroblastoma, glioblastoma, medulloblastoma, retinoblastoma, anaplastic large cell lymphoma, and small cell lung carcinoma [154, 159]. *MYCN* amplification is perhaps best known in neuroblastoma, in which it clearly correlates with poor outcome [154, 160].

Several studies also demonstrated *MYCN* amplification in RMS tumors [148, 161–166]. *MYCN* gene amplification occurs predominantly in ARMS [161–163] though recent studies revealed a low frequency of *MYCN* amplification in ERMS [148, 164, 165]. In the latest investigation, *MYCN* amplification was present in 25% of ARMS patient samples versus 6% of ERMS [165]. High MYCN expression levels significantly correlated with *PAX3/7-FOXO1* -positive ARMS tumors and poorer clinical outcome for ARMS patients [165]. Association of high MYCN expression with fusion gene positivity is consistent with previous findings that PAX3-FOXO1 increases MYCN mRNA expression [26, 148], suggesting that MYCN is a direct transcriptional target of the PAX3-FOXO1 oncoprotein. Moreover, ChIP-seq analysis revealed a PAX3-FOXO1 binding site downstream of the *MYCN* transcription start site, providing further evidence that PAX3-FOXO1 directly activates *MYCN* transcription [111].

As a transcription factor lacking enzymatic activity, perturbation of MYCN function is challenging. Numerous studies of the related MYC protein focused on disrupting MYC-MAX dimerization [152], which is conceptually a viable strategy. *In vitro* data provided proof of principle, but *in vivo* functionality of this approach remains to be evaluated [152]. Preclinical studies of *MYCN* antigene therapy, however, are promising [165]. In both ARMS and ERMS cell lines *in vitro,* treatment with an antigene peptide nucleic acid (PNA-*MYCN*) oligonucleotide that specifically inhibited *MYCN* mRNA expression abrogated proliferation and induced apoptosis [165].Antitumor activity of PNA-MYCN was especially marked and intriguing in ARMS; not only was PNA*-MYCN* effectiveness validated *in vivo* using a murine xenograft model, but also a novel interaction between MYCN and PAX3-FOXO1 was illuminated. Decreased MYCN levels resulted in reduced PAX3-FOXO1 expression, while MYCN overexpression led to elevated PAX3-FOXO1 levels [165]. This novel positive feedback mechanism has profound potential for therapeutic exploitation in ARMS, as anti-MYCN therapy will directly inhibit MYCN, thereby indirectly suppressing PAX3-FOXO1 as well. Like killing two birds with one stone, one agent could kill two oncogenes. Thus, in refractory ARMS tumors, MYCN-directed therapy may provide a surrogate strategy for PAX3-FOXO1 inhibition [167].

5. Conclusion

Although recurrent chromosomal translocations are uncommon in solid tumors, they are characteristic of approximately one-third of all sarcomas [6]. Studies of non-random chromosomal translocations and their resultant fusion genes have expanded our understanding of sarcoma biology, facilitated diagnosis and prognosis, and underscored the

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value of developing therapies to target oncogenic fusion genes. Using PAX3-FOXO1 in ARMS as a paradigm, ample evidence advocates two broad approaches for the treatment of translocation-associated sarcomas: 1) targeted therapies directed against the oncogenic chimera itself, and 2) therapeutic strategies targeting downstream genes activated by the fusion oncoprotein.

6. Expert Opinion

The uniqueness of *PAX3-FOXO1* expression to ARMS is a double-edged sword—on one hand, it is advantageous for PAX3-*FOXO1*-directed therapies, conferring cytotoxicity specifically to cancer cells. On the other hand, however, PAX3-FOXO1 is exclusively expressed in ARMS [1, 7], a rare pediatric tumor. The same scenario is mirrored in other translocation-associated sarcomas, including *ASPSCR1-TFE3* in alveolar soft part sarcoma and *FUS-DDIT3* in myxoid liposarcoma [1].

Although targeting specific oncogenic chimeras is a viable therapeutic approach, the rare tumor context in which these fusion genes are expressed presents considerable challenges. Currently, despite more than 800 new anticancer drugs estimated to be in clinical development for adult tumors, the biopharmaceutical industry does not conduct preclinical research and development for rare cancers [168]. From an economic perspective, the rationale of pharmaceutical companies is uncomplicated: drugs indicated for a rare malignancy have a smaller market and will therefore garner less profit compared to a more prevalent tumor. Drug research and development for rare cancers, such as ARMS and other translocation-associated sarcomas, is thus relegated to the academic sector funded by federal, foundation, and private grants. However, even if the pharmaceutical industry were to extend studies to include rare malignancies, patient enrollment in clinical trials would remain a major impediment to drug evaluation. Common cancers benefit by virtue of a greater patient pool, whereas resources for uncommon tumors are inherently limited, with accrual taking years rather than months. To compensate for the small patient population, clinical trials examining treatment for rare malignancies must recruit patients from numerous sites, which is the impetus for creation of cooperative clinical oncology trials groups such as the Children’s Oncology Group.

While efforts to develop targeted therapies against fusion proteins should not be abandoned, one must also be cognizant of molecular targets that are applicable to a broader tumor landscape. This review has highlighted FGFR4, MET, and MYCN as examples of such targets, but numerous other oncogenes, including IGF1R, PDGFR, VEGFR, PI3K, and MTOR (Table 1), are relevant to both rare, translocation-associated sarcomas [2] and to more common cancers. Such oncogenes may be found not only as genes downstream of the fusion oncoproteins but also potentially related to genetic events that cooperate with the fusion oncoproteins. By focusing on more generalizable molecular targets, the paradigm shifts to regard therapies not as tumor-specific, but as target-specific. With a higher likelihood for investment and a larger patient population from which to enroll for clinical trials, this approach provides the potential for including more rare malignancies in trials of mainstream targeted cancer therapeutics.

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Abbreviations

ARMS	Alveolar rhabdomyosarcoma
CDK1/2	Cyclin-dependent kinase 1/2
ChIP	Chromatin immunoprecipitation
CK1/2	Casein kinase 1/2
CNR1/CB1	Cannabinoid receptor 1
COL1A1-PDGFB	Collagen type I alpha 1-Platelet-derived growth factor beta
CPT1A	Carnitine palmitoyltransferase 1A
CTL	Cytotoxic T cell lymphocytes
DFSP	Dermatofibrosarcoma protuberans
EGFR	Epidermal growth factor receptor
ERMS	Embryonal rhabdomyosarcoma
EWSR1-FLI1	Ewing's sarcoma breakpoint region 1-Friend leukemia virus integration 1
FGFR	Fibroblast growth factor receptor
FISH	fluorescence in situ hybridization
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
IGF2	Insulin-like growth factor 2
IGF1R	Insulin-like growth factor 1 receptor
IL-2	Interleukin-2
MHC	Major histocompatibility complex
MTOR	Mammalian target of rapamycin
NCOA1/2	Nuclear receptor coactivator 1
ODC	Ornithine decarboxylase
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3-kinase

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PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PKC	Protein kinase C
PNA	Peptide nucleic acid
RMS	Rhabdomyosarcoma
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
TERT	Telomerase reverse transcriptase
TK	Tyrosine kinase
VEGFR	Vascular endothelial growth factor receptor

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* of importance

** of considerable importance

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7. Article Highlights

- Although recurrent chromosomal translocations are typically rare in solid tumors, they are associated with approximately one-third of all sarcomas.
- The majority of recurrent chromosomal translocations in sarcomas generate oncogenic chimeric transcription factors, which aberrantly transactivate target gene expression.
- Using the PAX3-FOXO1 fusion oncoprotein in ARMS as an example, evidence is discussed that constructs a conceptual framework of therapeutic strategies to target not only the fusion product itself, but also the downstream products mediating fusion protein oncogenicity.
- Most fusion genes are expressed in rare tumors, creating considerable challenges for drug development and clinical evaluation. Therefore, an important additional approach is to exploit downstream targets in these translocation-associated sarcomas that are also applicable to more common cancer categories.

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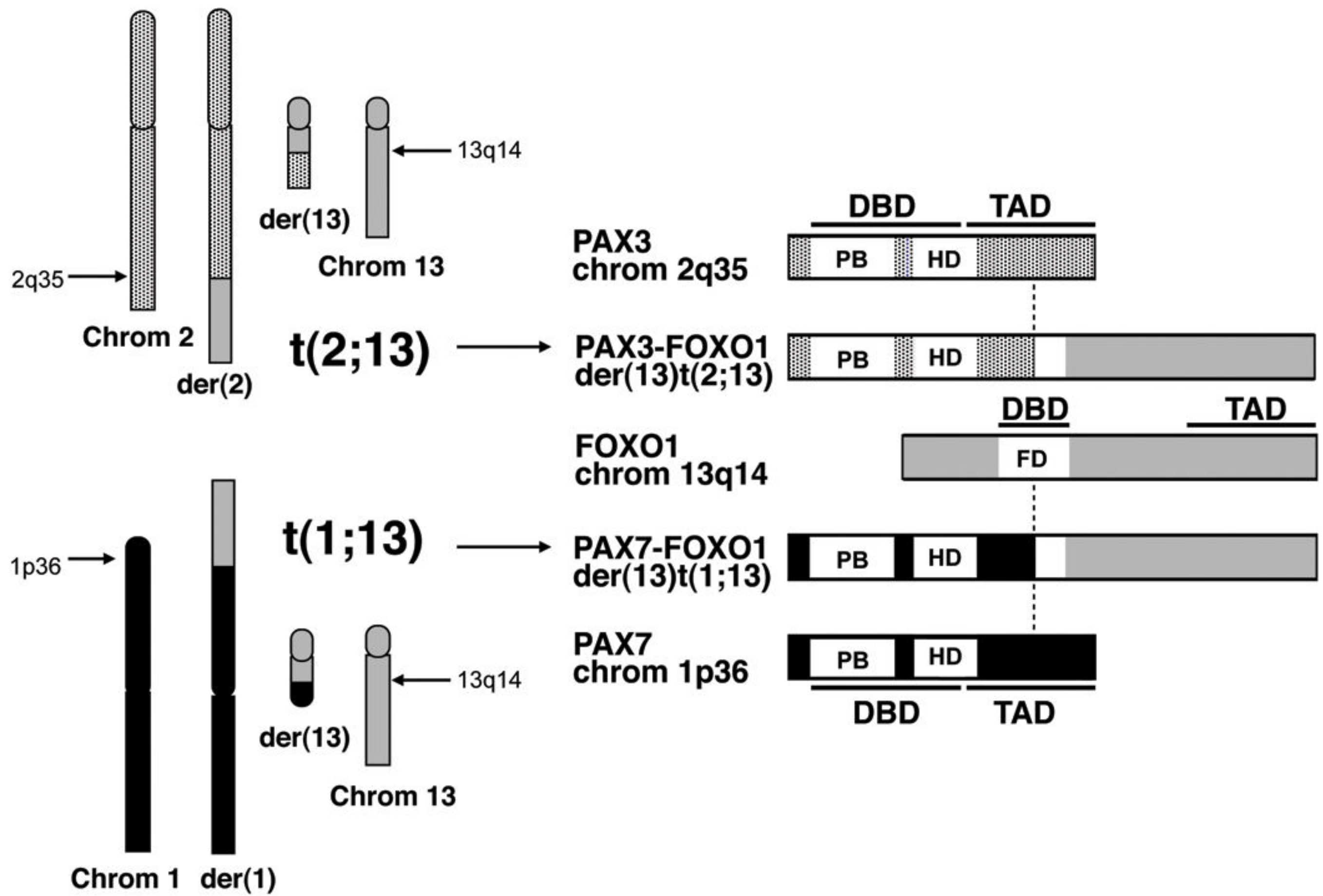

Figure 1.
Schematic depiction of t(2;13)(q35;q14) and t(1;13)(p36;q14) chromosomal translocations and resultant PAX3/7-FOXO1 chimeric fusion products. The vertical dashed line denotes the fusion point. DBD: DNA binding domain; FD: Forkhead domain; HD: Homeobox domain; PB: Paired box; TAD: Transcriptional activation domain.

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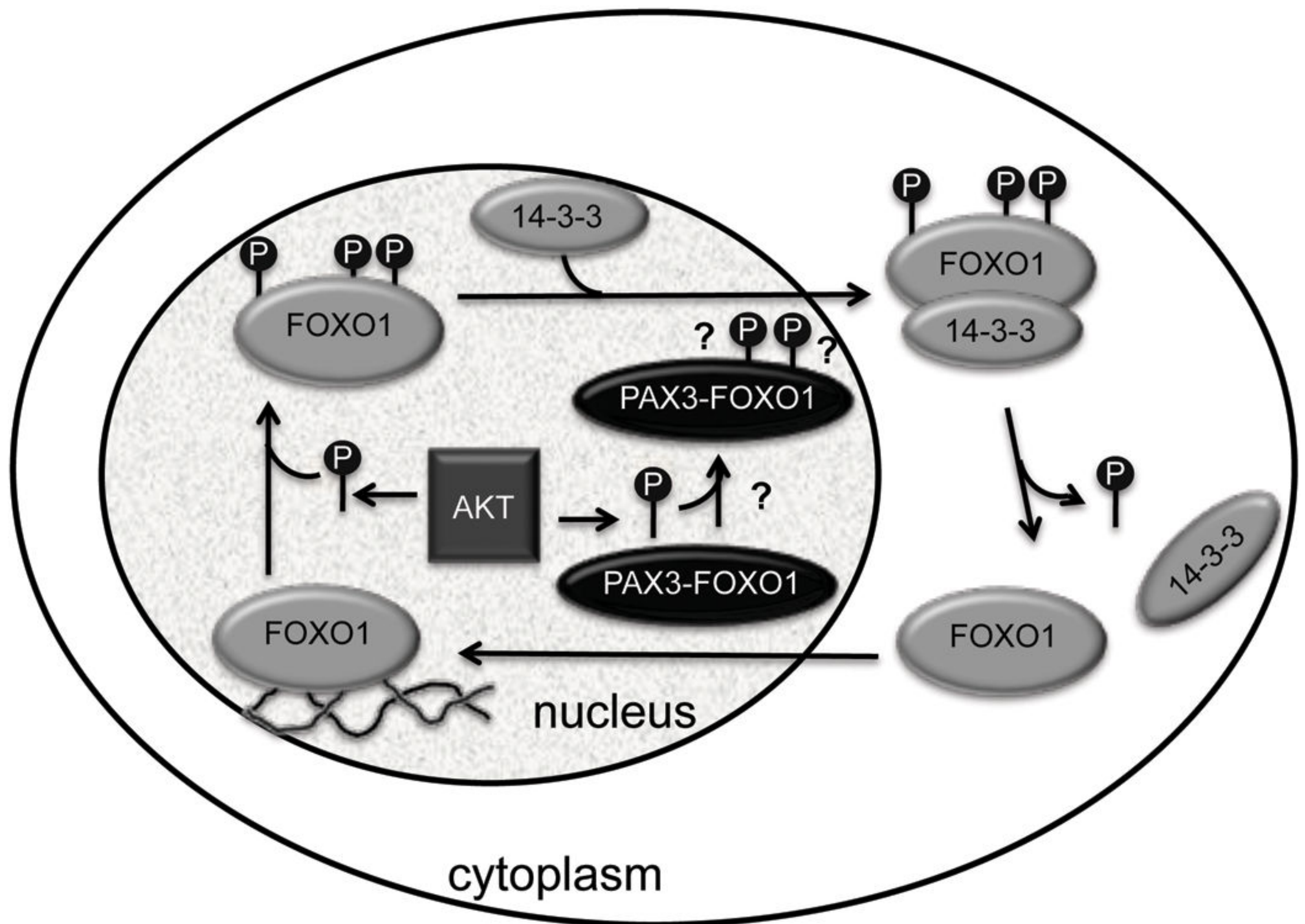

Figure 2.

Phosphorylation-mediated regulation of FOXO1, but not PAX3-FOXO1, subcellular localization. Wild-type FOXO1 contains three evolutionarily conserved AKT phosphorylation sites (P). AKT-driven phosphorylation at these FOXO1 residues promotes 14–3-3 protein docking and binding, resulting in inactivation of FOXO1 transcriptional activity via cytoplasmic sequestration. PAX3-FOXO1 is resistant to AKT-mediated regulation by phosphorylation as evidenced by its constant nuclear localization.

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Table 1.

Therapeutic targets implicated in ARMS and additional cancer categories.

Target	Agent	Alteration in RMS	Alteration/Implication in Other Tumor Types
CNR1/CB 1	AM251; HU210; Delta(9)-tetrahydro-cannabinol[169, 170]	Overexpression; Induced by PAX3-FOXO1 [169, 170]	Invasion in breast cancer; migration in kidney [171, 172]
CPT1A	Etomoxir [173]	Direct PAX3-FOXO1 transcriptional target [174]	Proliferation and motility in lung [175] and prostate cancer [175, 176]
FGFR4	PD173074; AZD4547; AZ12908010 [109112,123, 124]	Overexpression in RMS; Direct PAX3-FOXO1 transcriptional target; Association with PAX3-FOXO1-positive ARMS and reduced overall survival; Activating mutations found in 7.5% of RMS tumors (mostly ERMS) [95, 105–109, 111]	Overexpression in several malignancies (e.g., breast, gynecologic, lung, liver, pituitary, prostate, and pancreas) [116–122, 177]
IGF1R	Cixutumumab; Figitumumab; Teprotumumab [1, 2]	Overexpression; Association with aggressive behavior and reduced failure-free survival in ARMS [2, 178]	Overexpression in numerous human cancers (e.g., breast, colorectal, melanoma, liver, prostate, and sarcoma) [179, 180]
MET	Crizotinib; Tivantinib; OA-5D5; DN30; K252; SU11274; PHA665752; PF2341066; XL880; MK2461; MP470; SGX523; JNJ38877605 [1, 2, 128]	Association with migration, invasion, and metastasis; Correlation of high MET expression with advanced stage, worse outcome, ARMS histology, and PAX3-FOXO1 expression; Direct PAX3-FOXO1 transcriptional target [142–147, 149, 150]	Overexpression in almost all carcinoma types (e.g., breast, colorectal, hepatocellular, oral squamous cell, ovarian, pancreatic, prostatic, renal cell, and thyroid) [128, 131–141]
MTOR (mTOR)	Everolimus; Ridaforolimus; Sirolimus; Temsirolimus [1, 2]	Strong association between activation of mTOR signaling components and poor failure-free or overall survival [181, 182]	Deregulation in several cancers (e.g., hamartoma syndromes, lymphomas, breast, and melanoma) [183–187]
MYCN	PNA-M7CW[165]	Amplification, most frequently in ARMS; Correlation between high MYCN expression and *PAX3/7-FOXO1* -positive ARMS tumors and poorer clinical outcome; Direct PAX3-FOXO1 transcriptional target [111,148, 161–166]	Aberrant expression of MYCN as a result of *MYCN* amplification detected in several malignancies (e.g., neuroblastoma, glioblastoma, medulloblastoma, retinoblastoma, anaplastic large cell lymphoma, and small cell lung carcinoma) [154, 159]
PDGFR	Imatinib; Olaratumab; Sorafenib; Dasatinib; Sunitinib; Axitinib; Pazopanib [1, 2]	Overexpression of PDGFR-A in ARMS and ERMS; Association between PDGFR expression and decreased survival [178, 188]	Aberrant expression or overexpression in a variety of cancers (e.g., glioma, breast, ovarian, prostate, and lung) [189–192]
PI3K	GSK1059615; BEZ235 [2]	Putative overexpression/gain-of-function mutations based on downstream activation of AKT-mTOR axis [79, 181]	*PIK3CA* mutations in several malignancies (e.g., breast, colon, endometrial, glioblastoma, ovarian); *PIK3CA* amplification in many other tumors (e.g., head and neck, squamous cell lung carcinoma, cervical, gastric, and esophageal) [193]
VEGFR	Bevacizumab; Brivanib; Cediranib; Sorafenib; Sunitinib; Axitinib; Pazopanib [1, 2]	Higher VEGFR expression in ARMS versus ERMS implicated in metastatic phenotype of ARMS [194]	Aberrant expression or overexpression in vasculature of numerous solid tumors (e.g., breast, ovarian, colorectal, and lung) [195, 196]

ARMS: Alveolar rhabdomyosarcoma; CNR1/CB1: Cannabinoid receptor 1; CPT1A: Carnitine palmitoyltransferase 1A; ERMS: Embryonal rhabdomyosarcoma; FGFR4: Fibroblast growth receptor 4; IGF1R: Insulin-like growth factor 1 receptor; MTOR: Mammalian target of rapamycin; PDGFR: Platelet-derived growth factor receptor; PI3K: Phosphatidylinositol 3-kinase; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; PNA: Peptide nucleic acid; RMS: Rhabdomyosarcoma; VEGFR: Vascular endothelial growth factor receptor