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Genetics, epigenetics and redox homeostasis in rhabdomyosarcoma: Emerging targets and therapeutics

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ABSTRACT

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma accounting for 5–8% of malignant tumours in children and adolescents. Children with high risk disease have poor prognosis. Anti-RMS therapies include surgery, radiation and combination chemotherapy. While these strategies improved survival rates, they have plateaued since 1990s as drugs that target differentiation and self-renewal of tumours cells have not been identified. Moreover, prevailing treatments are aggressive with drug resistance and metastasis causing failure of several treatment regimes. Significant advances have been made recently in understanding the genetic and epigenetic landscape in RMS. These studies have identified novel diagnostic and prognostic markers and opened new avenues for treatment. An important target identified in high throughput drug screening studies is reactive oxygen species (ROS). Indeed, many drugs in clinical trials for RMS impact tumour progression through ROS. In light of such emerging evidence, we discuss recent findings highlighting key pathways, epigenetic alterations and their impacts on ROS that form the basis of developing novel molecularly targeted therapies in RMS. Such targeted therapies in combination with conventional therapy could reduce adverse side effects in young survivors and lead to a decline in long-term morbidity.

Abbreviations: AKT, Protein Kinase B; ALK, Anaplastic Lymphoma Kinase; AP1, Activator Protein 1; ARMS, Alveolar Rhabdomyosarcoma; ATP2A3, ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+; BCOR, BCL6 Corepressor; BET, Bromodomain and extra-terminal; BIO, 6-bromoindirubin-3′-oxime; BRAF, Proto-oncogene B-Raf and V-Raf Murine Sarcoma Viral Oncogene Homolog B; BRD, BET-containing Proteins; CDK, Cyclin Dependent Kinase; CK1α, Caesin Kinase 1 α; COX7A1, Cytochrome c Oxidase Polypeptide 7A1; CSC, Cancer Stem Cell; DDAH1, Dimethylarginine Dimethylaminohydrolase 1; DHH, Desert Hedgehog Protein; DLL1, Delta Like 1; DNMT, DNA Methyltransferase; DZNep, Deazaneplanocin A; EGFR, Epithelial Growth Factor Receptor; EMT, Epithelial to Mesenchymal Transition; ERMS, Embryonal Rhabdomyosarcoma; ERK1/2, Extracellular Signal Regulated Kinase ½; EZH2, Enhancer of Zeste Homolog 2; FDA, US Food and Drug Administration; FGFR, Fibroblast Growth Factor Receptor; GLI, Glioma Associated Oncogene; GLRX, Glutaredoxin; GSI, Gama Secretase Inhibitor; GSK3, Glycogen Kinase synthase; GTP, Guanosine-5′-triphosphate; HAT, Histone Acetyltransferase; HES1, Hairy and Enhancer of Split-1; HEY1, Hairy and Enhancer of Split Related with YRPW Motif Protein 1; HHIP, Hedgehog Interacting Protein; HIF-1 α, Hypoxia Inducible Factor 1 α; IC, Intracellular; ICN1, Intracellular Notch Domain 1; IGF, Insulin-like Growth Factor; IGF-1R, Insulin-like Growth Factor1 Receptor; IHH, Indian Hedgehog Protein; JAG1, Jagged 1; JNK, Janus Kinase 1; KMT1A, K-methyltransferase 1 A; LiCl, Lithium Chloride; LKB1, Threonine Kinase; LRP, Low Density Lipoprotein Receptor Related Protein; MCU, Mitochondrial Calcium Uniporter; MEF2C, Myocyte Enhancer Factor 2C; MEK, Mitogen Activated Protein Kinase; MICU1, Mitochondrial Calcium Uptake 1; MOB1, Mps One Binder Kinase Activator-like 1; MST1, Macrophage Stimulating 1; mTOR, (PI3K)-AKT-mammalian target of rapamycin; MYOD1, Myoblast Determination Protein; MYF5, Myogenic Factor 5; NICD, Notch Intracellular Domain; NOSIP, Nitric Oxide Synthase Interacting Protein; NOS1, Nitric Oxide Synthase 1; PAX3/7-FOXO1, Paired Box Protein 3/7-Forkhead Box Protein O1; PcG, Polycomb group; PCP, Planar Cell Polarity; PDGF, Platelet Derived Growth Factor; PDX, Patient Derived Xenograft; PI3K, Phosphatidylinositol-3-Kinase; PPP, Picropodophyllin; PRC2, Polycomb Repressive Complex 2; PTCH, Patched; PTK2, Protein Tyrosine Kinase 2; P38MAPK, P38 Mitogen Activated Protein Kinase; RASSF4, Ras Association domain family member 4; RBPJ, Recombining Binding Protein Suppressor of Hairless; RHOA, Ras Homolog Gene Family Member A; ROS, Reactive Oxygen Species; SFK, YES/SRC family tyrosine kinase; S6K1, S6 Kinase 1; SFRP, Secreted Frizzled Related Proteins; SMO, Smoothened; SUFU, Suppressor of Fused; SHH, Sonic Hedgehog; SAHA, Suberoylanilide Hydroxamic Acid; SP, Specificity Transcription Factor; SETD2, SET Domain Containing 2; SOD, Superoxide Dismutase; SOX2, Sex determining Region Y-box 2; TCF/LEF, Transcription factor/Lymphoid Enhancer Binding Factor 1; TPA, 2-O-tetradecanoylphorbol-13-acetate; TPC, Tumour Propagating Cells; TSA, Trichostatin A; TXNDC12, Thioredoxin Domain-containing Protein 12; TXN, Thioredoxin; VAC, Vincristine, Actinomycin D and Cyclophosphamide; VEGF, Vascular Endothelial Growth Factor; VANGL2, Van Gogh-like 2; WHO, World Health Organization; YAP, Yes Associated Protein 1; 5-aza-dc, 5-aza-2′-deoxycytidine; 2-ME, Methoxyestradiol

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1. Introduction

Rhabdomyosarcoma (RMS) is a paediatric cancer of skeletal muscle that arises due to the failure of skeletal myoblasts to undergo differentiation. RMS cells express the key myogenic protein MyoD, yet fail to irreversibly exit the cell cycle and complete myogenesis.

RMS is histologically classified by the World Health Organization (WHO) into four different subtypes: embryonal (ERMS), alveolar (ARMS), spindle cell/sclerosing, and pleomorphic [1]. The two major subtypes are ERMS and ARMS that account for 70% and 20% RMS respectively. In terms of gene-expression, RMS can be broadly classified as *PAX3/7-FOXO1* fusion-positive or fusion-negative tumours that closely associate with tumour progression, prognosis and clinical features. Approximately 80% of ARMS tumours are PAX3/7-FOXO1 fusion positive and this translocation results in higher propensity to metastasize to the bone marrow [2]. Expression of the fusion protein promotes proliferation through the expression of receptor tyrosine kinase molecules such as FGFR4, ALK and MET [3–5]. Fusion-negative ERMS possess heterogeneous histology with a complex karyotype, loss of heterozygosity, and single nucleotide point mutations [6]. Mutations in Ras, receptor tyrosine kinase or phosphoinositide-3 kinase complex are most commonly found. Despite significant advances in the understanding of the cellular and molecular mechanisms underlying the disease, no targeted drug therapy is available for these cancers.

RMS patients are stratified for diagnosis and treatment according to the histology and the site of occurrence of the tumour. The current gold standard treatment for RMS is a multimodal therapeutic strategy that was established in the 1970s (Fig. 1). Chemotherapeutic drugs vincristine, actinomycin D and cyclophosphamide (VAC) form the backbone for the treatment along with surgery and radiation. Vincristine and actinomycin D are used for low risk RMS patients to avoid large cumulative alkylator exposure of cyclophosphamide that has been associated with secondary malignancies and sterility [7–9]. Patients with intermediate risk are prescribed with VAC in combination with other agents such as etoposide, ifosfamide, cisplatin, irinotecan, topotecan, doxorubicin or intensifying cyclophosphamide to improved clinical outcome [10–12]. RMS patients with *PAX3-FOXO1* translocation are often classified as high-risk and require more intensive chemotherapy backbone using vincristine, doxorubicin and cyclophosphamide that is alternated with ifosfamide and etoposide. This regimen considerably improved the prognosis of RMS patients. Fig. 2 gives a snapshot of molecular drugs that have currently shown efficacy in preclinical and clinical trials in the two major subtypes of RMS. However, there have been meagre improvements to treatment options since then, and cure rates have stagnated due to the lack of targeted therapies.

Treatment modalities based on site of tumour and histological subtype can be ineffective due to varying genetic expression profiles within RMS subtypes. For instance, *PAX3/7-FOXO1* fusion commonly found in ARMS is linked to aggressive tumour progression and intensive multimodal treatment. However, a small proportion of fusion negative patients have prognosis and molecular genetics similar to ERMS. Thus, subjecting them to intense treatment would expose them to unnecessary risk of late effects especially in development. Since RMS occurs in children under the age of 15 [13], it is also essential to consider harmful post-treatment effects including profound functional deficits, organ toxicities and secondary cancers [14] that may manifest later in life. The use of alkylating agents like cyclophosphamide and ifosfamide as chemotherapy drugs have been linked to secondary malignancies. Dose-dependent effects of alkylating agents on testicular function and fertility been reported in male patients [7,8,10] while female patients are known to have an increased risk of premature ovarian failure and infertility [15,16]. Other possible side-effects include peripheral nervous system toxicity and cardiac dysfunction [17–19]. The inadequacy of current standard of care is evident in less than 30% survival rate for patients with fusion positive or overtly metastatic RMS even with most advanced multimodal therapies [20–23]. There is a clear unmet need to develop novel, targeted, and safer therapies for high risk RMS.

The improved understanding of genetic and epigenetic alterations in RMS, as well as advancement in techniques to interrogate molecular alterations has opened avenues to develop molecular therapies [23–25]. For instance, genome-wide DNA methylation has revealed RMS subtype-specific aberrant DNA methylation in genes associated with tissue development, differentiation and oncogenesis. These results suggest that RNA and DNA methylation signatures that distinguish RMS subtypes could serve as therapeutic targets [26,27]. Targeting

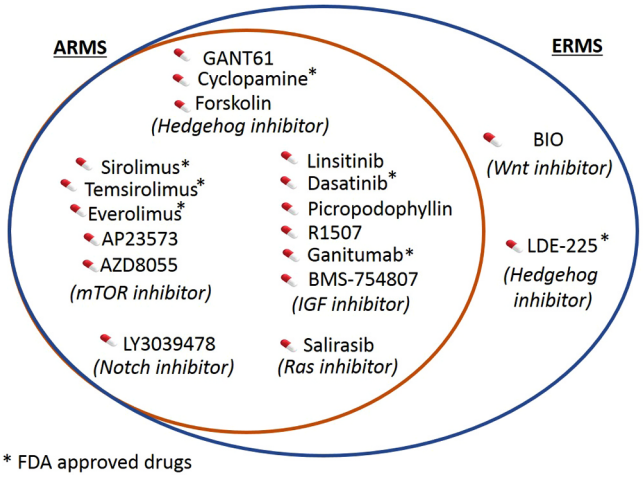

Fig. 2. Molecularly targeted drugs for RMS. List of drugs targeting various de-regulated molecular pathways that have shown effects in inhibiting tumour progression either in ARMS or ERMS or both RMS subtypes.

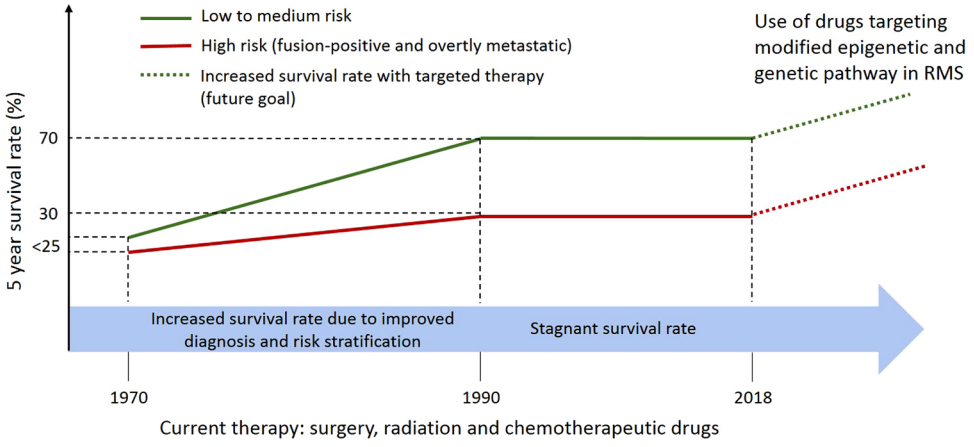

Fig. 1. 5-year survival rate of RMS patients from 1970s. The 5-year survival rate of RMS patients increased from 1970s to 1990s with improved molecular understanding resulting in better diagnosis and risk stratification. The survival rate of RMS patients has been stagnant since 1990s with no improvement to treatment. The gold standard of care remains the use of chemotherapeutic drugs with surgery and radiation.

Table 1
Epigenetic and genetic drugs used as monotherapy or in combination in RMS.

Epigenetic and Genetic Drugs	Single agent effects	Combination therapy	References
SAHA (HDAC inhibitor)	Induction of apoptosis, differentiation and inhibition of self-renewal, invasion and migration.	Radio-sensitisation in ERMS, chemosensitisation of RMS to doxorubicin, etopiside.	[16–18]
GSK126 (EZH2 inhibitor)	Promote myogenic differentiation in ERMS.	Synergistic effect on differentiation with 12-O-tetradecanoylphorbol-13-acetate (TPA) in ERMS.	[19]
GSK690, E917 (LSD1 inhibitor)	Little cytotoxicity.	Induce mitochondrial apoptosis when combined with HDAC inhibitors SAHA, JNJ-26481585.	[20]
5-aza-dc (DNMT inhibitor)	Decrease proliferation and migration, induce differentiation and reduced tumour development.	Synergistically prevent tumour formation when combined with HDAC inhibitor valproic acid.	[21–23]
R1507 (IGF-1R inhibitor)	Inhibition of cell growth.	Combination with Dasatinib (multi targeted tyrosine kinase inhibitor) synergistically inhibited cell growth.	[24]
LDE-225 (Hedgehog inhibitor)	Induction of apoptosis.	Decreased chemoresistance against irinotecan by decreasing self-renewal.	[25,26]

The effect of drugs in RMS when used as a single agent along with their synergistic effects in combination with other drugs.

epigenetic de-regulations may therefore be significant in development of novel therapies [14]. Moreover, as epigenetic changes are closely linked to chemoresistance, use of epi-drugs in combination with conventional therapies may solve the current stagnant treatment efficacies. Table 1 lists drugs targeting genetic and epigenetic pathways that have shown efficacy either as a single agent and/or in combination with other drugs. In addition, high throughput drug screening has shown that reactive oxygen species (ROS) plays a role in tumour progression which provides an additional avenue for therapeutics. [28–38]

In this review we examine key signalling pathways, epigenetic alterations, and the altered redox balance in RMS to explore the plausibility of developing molecular targeted therapies. We also discuss the potential of epi-drugs and redox modulators that are currently undergoing clinical trials or have shown efficacy in pre-clinical trials.

2. Genetic alterations in RMS

From molecular and genomic studies, it is evident that these tumours arise from aberrant signalling and growth pathways. Genetic alterations in RMS lead to deregulated signalling pathways that promote tumour progression. Small-molecule inhibitors or biologics that target these pathways provide translational opportunities.

2.1. Notch signalling pathway

Notch signalling in mammals consists of four Notch transmembrane receptors (Notch1–4) and five Notch ligands [Jagged (JAG) 1 and 2 and Delta-like (DLL) 1, 3 and 4]. Notch signalling is activated upon ligand binding to the Notch receptors. The receptor undergoes proteolytic cleavage at two sites. The last cleavage is mediated by the presenilin-containing gamma-secretase complex, which results in the formation of the Notch intracellular domain (NICD). NICD translocates to the nucleus to transcriptionally activate target genes.

In adult myogenesis, Notch signalling is upregulated in activated satellite cells. This upregulation promotes transition into proliferating myoblasts [39] and prevents differentiation [40]. Consistently, the Notch pathway is activated in RMS. Notch1, Notch2 and Notch 3 are overexpressed along with downstream targets Hes1 and Hey1 in *PAX3-FOXO1* fusion positive ARMS cell lines. The expression of Hes1 and Hey1 coincides with invasive capacity. Treatment with gamma secretase inhibitors (GSI) to block Notch activation resulted in significant reduction in migratory and invasive capacity of tumour cells with no effect on cell cycle progression or apoptosis [41]. ERMS tumours also showed a high nuclear (active) Notch1. Both knockdown of Notch1 and Hey1 showed inhibition of cell growth with increased myogenin expression, and GSI phenocopied the effect of Notch suppression. *In vivo*, genetic and pharmacologic inhibition of Notch blocked tumour growth as well [42]. In addition, Notch3 was found to be activated by the ligands JAG1 and DLL1. Notch3 downregulation decreased proliferation and inhibited tumour growth *in vivo* [43]. Conversely, overexpression of the Notch3 intracellular (IC) domain in both ERMS and ARMS cell lines increased proliferation through ERK1/2 phosphorylation. The anti-proliferative effect of GSI was rescued in part by Notch3 IC overexpression. *In vivo*, Notch3 IC overexpressing cells showed a higher tumorigenic potential than controls. Consistently, a higher number of Notch3-Hes1 and Ki67 positive cells were found in both ERMS and ARMS primary tumours compared to normal skeletal muscle [44]. Interestingly, in a transgenic zebrafish model of ERMS, a molecularly distinct subpopulation of tumour propagating cells (TPC) that self-renew and sustain tumour growth was identified. Notch1 activation was found to expand TPC by dedifferentiating differentiated ERMS cells. Consequently, Notch1 knockdown led to a reduction in self-renewal capacity and significantly reduced tumour growth and maintenance in ERMS xenografts. Notch1 was found to regulate TPC by upregulating SNAIL1 that stimulates self-renewal and expansion of TPC, and suppresses myogenic differentiation by silencing MEF2C [33]. LY3039478, an oral Notch inhibitor has been recently studied in a multiple part phase I trial to determine its safety and efficacy in patients with soft tissue sarcoma, and also in patients with gastrointestinal stromal tumours. LY3039478 showed a modest clinical activity and manageable safety profile with the most common adverse side effects being diarrhoea, nausea, vomiting and decreased appetite. In general, Notch1 positive tumours showed a higher response than Notch1 negative tumours [45,46].

2.2. IGF signalling pathway

Insulin-like growth factor (IGF) is an important regulator of muscle growth, regeneration, hypertrophy and differentiation [47–49]. Several components of IGF signalling such as IGF-I and IGF-II increase during proliferation and maturation of myoblasts and myotubes respectively [48]. In RMS, inhibition of IGF signalling decreases cell growth *in vivo* [50]. IGF receptors belong to a larger class of tyrosine kinase receptors, many of which such as IGF-1R are known to be overexpressed or mutated in RMS [50–52]. Furthermore, there is a loss of imprinting of the IGF-2 locus in both ERMS and ARMS. In fusion-positive RMS tumours, IGF-2 is upregulated by *PAX3-FOXO1* and activates IGF-2 pathway [53]. IGF-2 has also been shown to be overexpressed and upregulated in RMS mouse models [54]. Numerous IGR-1R inhibitors such as linsitinib, BMS-754807 and picropodophyllin (PPP) were developed and many pre-clinical studies showed optimistic results. Tarnowski et al. [55] demonstrated that PPP effectively inhibit RMS tumour growth both *in vitro* and *in vivo*. Both monotherapy and combination therapy with chemotherapeutic drugs or CDK inhibitors were found effective in pre-clinical studies [55–58]. Since then, more than 10 IGF-1R inhibitors have entered clinical trials [59]. However, the results from phase II and III clinical studies for IGF-1R inhibitors were disappointing. This may be due to the complexity of the IGF-IR, presence of compensating

Table 2
Drugs that have therapeutic effects through regulation of ROS.

Drugs	Mechanism	Effect on ROS	Current application	References
Carfilzomib	Proteasome inhibitor that results in growth arrest and apoptosis of tumour cells	Increases oxidative stress and induces mitochondrial cell death	Chemotherapeutic drug for multiple myeloma	[136]
Auranofin	Radiosensitizer	Targets thioredoxin reductase and induces overproduction of ROS	Rheumatoid arthritis	[137]
Cerivastin	Inhibits HMG-CoA-reductase and potentially decreases proliferation and invasion	Targets RAS protein trafficking and increases ROS production	Hypercholesterolemia, Rhabdomyolysis	[138–140]
Alvocdib	Multi-serine threonine cyclin-dependent kinase inhibitor	Increases ROS production	Acute myeloid leukaemia	[141]
Ouabain	Cardiac glycoside that inhibits ATPase sodium-potassium ion pump	Increases mitochondrial ROS production	Hypotension, cardiac arrhythmia	[142,143]

List of drugs that have an effect on ROS production in cancer cells that may potentially be used in combination therapy.

pathways, resistance development and difficulty in patient selection [59–61].

One of the proposed pathway that is involved in the resistance to IGF-1R inhibitors [R1507 (IGF-1R antibody) and BMS-754807 (IGF-1R kinase inhibitor)] is the upregulation of YES/SRC family tyrosine kinase (SFK) [36]. SFKs are non-receptor tyrosine kinases that promote proliferation, migration and invasion. SFKs are upregulated in various cancers [36]. Dual blockage of IGF-1R and SFK pathways is therefore proposed for RMS treatment [36]. Pre-clinical studies showed that combination of R1507 and dasatinib (multitargeted tyrosine kinase inhibitor) significantly inhibits tumour growth *in vivo* and failed to develop resistance even after 79 days of treatment [36]. Currently, phase I and II clinical trials of combination therapy with ganitumab (IGF-1R antibody) and dasatinib on ERMS and ARMS are on-going to overcome drug resistance seen with monotherapy.

2.3. PI3K/mTOR signalling pathway

One of the main pathways downstream of IGR-1R is the phosphatidylinositol 3′ kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) [62]. mTOR connects two major signalling pathways: PI3K and serine threonine kinase (LKB1), an energy-sensing pathway. mTOR exists in two different complexes, namely mTORC1 and mTORC2. Activation of the mTORC1 and the downstream effectors eukaryotic initiation factor 4E (4E-BP1) and protein S6 kinase 1 (S6K1) are important in protein synthesis, cell growth and proliferation. Some of their oncogenic targets include vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1α (HIF-1α) and cyclin D1 [63,64].

In RMS, upregulated IGF-1R, FGFR and EGFR signalling pathways lead to activation of mTOR signalling through PI3K and AKT [24,65–68]. High levels of phosphorylated AKT have been reported in RMS cell lines and primary tumours [69,70]. Hence, this pathway has been explored for therapeutic intervention.

Rapamycin (sirolimus) is an FDA approved natural inhibitor of mTOR in the mTORC1 complex that acts as an immunosuppressant. It also demonstrates promising inhibitory activity against tumour progression both *in vitro* and *in vivo* [52]. However, due to rapamycin's poor aqueous solubility and chemical stability, several synthetic derivatives including CCI-779 (temsirolimus), RAD001 (everolimus), AP23573 and AZD8055 were developed. Pre-clinical studies of AZD8055 showed evident anti-tumour activity again RMS and combinatorial treatment with ABT-737, a BH3 mimetic showed synergistic lethality [71]. Reduced growth of RMS tumours in mouse xenograft models was also reported [72].

2.4. RAS signalling pathway

The most common oncogenic mutation in fusion-negative RMS (mostly ERMS) is the RAS signalling pathway. Mutations in any one of the RAS isoforms, NRAS, HRAS or KRAS maintain the protein in its GTP bound state and result in elevated RAS signalling in RMS [6,73]. Despite the differences in molecular genetics, fusion-positive RMS (mostly ARMS) also exhibit disrupted receptor tyrosine kinase/RAS/PIK3CA axis through the translocation of the *PAX* gene and accumulation of mutations stemming from *PAX3/7-FOXO1* fusion [53,74].

Due to the prevalence of RAS mutations in various cancers, considerable effort has been directed at developing therapeutic interventions targeting RAS signalling. However, direct targeting of RAS remains a challenge and the only reported drug in clinical trial is salirasib, which targets KRAS. Salirasib, an oral KRAS inhibitor is in early phase I clinical trials for relapsed or refractory solid tumours. Salirasib has been found to be safe, well tolerated by patients and seems to prolong progression-free period [75]. However, more rigorous clinical trials need to be performed to evaluate the efficacy of salirasib in treating cancer or RMS specifically. Although direct targeting of RAS family is challenging, the use of small molecules that targets upstream

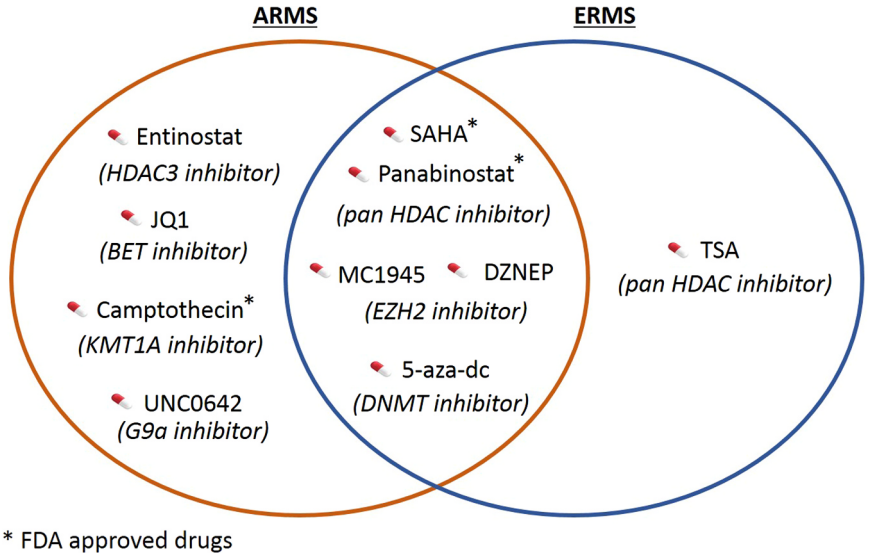

Fig. 3. Potential epigenetic drugs for RMS. List of drugs targeting epigenetic regulators that inhibit tumour progression in ARMS or ERMS or both subtypes.

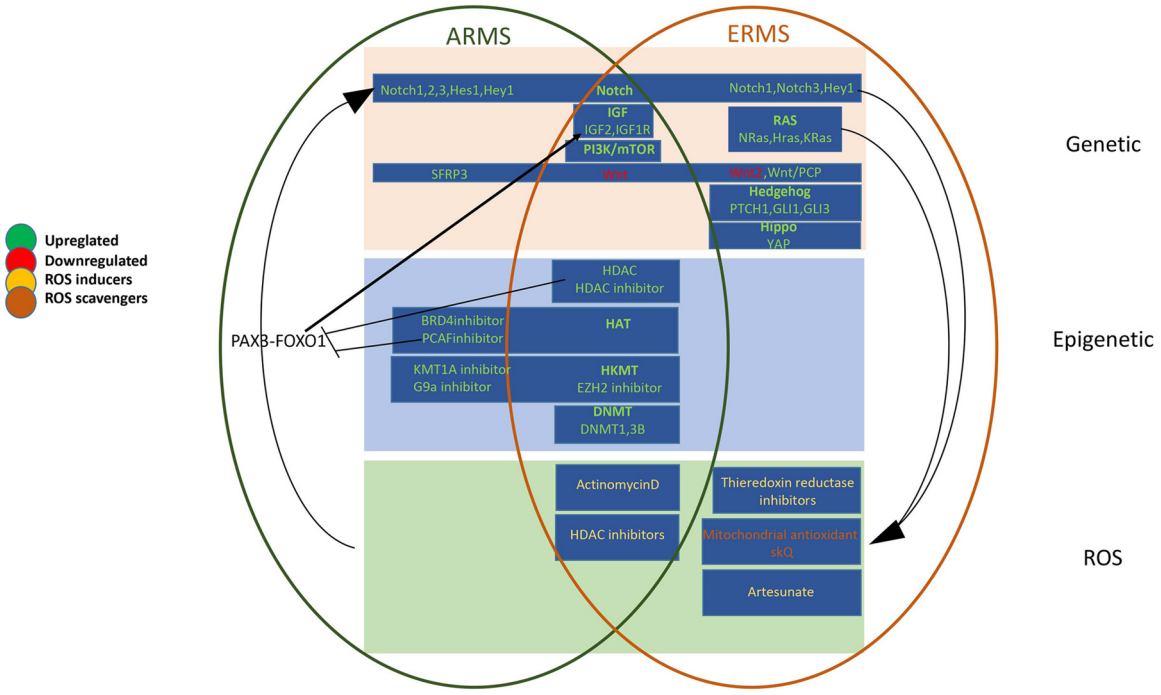

Fig. 4. Cross talk between genetic, epigenetic and ROS deregulation in RMS. Summary of crosstalk between the genetic, epigenetic and ROS deregulation in the two major subtypes of RMS.

or downstream molecules of RAS pathway are possible. Several BRAF inhibitors (vemurafenib, dabrafenib) and MEK inhibitor (trametinib) have gained US Food and Drug Administration (FDA) approval for treatment of some RAS-driven cancers such as metastatic melanoma [6].

2.5. WNT signalling pathway

Canonical WNT signalling is activated by binding of WNT ligands to the frizzled receptor and co-receptor LRP5/6. This results in activation of β-catenin, which translocates to the nucleus and aids transcription of TCF/LEF target genes. In the absence of Wnt ligands, β-catenin is phosphorylated by casein kinase I α (CK1α) and glycogen kinase synthase 3 beta (GSK3β), and marked for degradation [76].

The importance of WNT pathway in RMS was initially shown in p53-/-/c-fos-/- double mutant mice which develop ERMS tumours. Since c-fos: c-jun form the predominant AP1 complex, removal of c-fos results in an aberrant AP1 complex. Among the AP1 targets, WNT2 among other Wnt pathway genes were differentially regulated. Canonical WNT signalling and β-catenin were lower in ERMS compared to normal myoblasts. Inhibition of canonical Wnt signalling was also confirmed in ERMS cell lines [77]. Induction of Wnt pathway by the GSK3 inhibitor LiCl induced differentiation in ERMS, with no evidence of apoptosis. Similarly, recombinant WNT3A increased differentiation markers MyoD, MYF5 and myogenin in both ARMS and ERMS cell lines.

A high throughput screen for drugs that induce differentiation in ERMS identified six different classes of inhibitors: GSK3, RAF/MEK protein kinase, PI3-kinase/AKT protein kinase, Hedgehog pathway and HDACs along with DNA damaging agents. Of these, *in vivo*, the GSK3 inhibitor BIO (6-bromoindirubin-3′-oxime) was the only hit besides the

pan-HDAC inhibitor Trichostatin A (TSA) to show growth inhibitory effects and induction of differentiation. The induction of differentiation by BIO seemed to be limited to ERMS. BIO activated canonical WNT signalling in ERMS with a paradoxical decrease in mTOR signalling. This is surprising since inhibition of GSK3β activates mTOR signalling indicating that the induction of differentiation in ERMS is mediated through Wnt signalling. GSK3β inhibitors were also able to decrease TPCs in a zebrafish ERMS model as well as reduce self-renewal in ERMS cell lines [76]. Transcriptomic analysis of human myoblasts with or without *PAX3-FOXO1* revealed that the secreted inhibitor SFRP3 was upregulated in all *PAX3-FOXO1* fusion positive ARMS cell lines. Inhibition of SFRP3 decreased proliferation and induced apoptosis. In mouse xenografts models, knockdown of SFRP3 resulted in a decrease in tumour volume and weight. *In vivo*, SFRP3 knockdown tumours showed increase in myogenic differentiation genes MyoD, myogenin and Myf5. SFRP3 blockade in combination with vincristine resulted in tumour regression in xenograft models indicating relevance of SFRP3 inhibitors in combination therapy [78].

While the role of canonical WNT signalling has been studied in RMS, very little is known about non-canonical WNT signalling. Van Gogh-like 2 (VANGL2) protein, which is a core regulator of non-canonical WNT/planar cell polarity (WNT/PCP) pathway was found to have a role in the TPC population self-renewal in both human and zebrafish RMS models. VANGl2 knockdown resulted in decreased TPC population with an increase in tumour proliferation and induction of differentiation. This effect was shown to be mediated by RhoA [79].

2.6. Hedgehog signalling pathway

The Hedgehog pathway (HH) is activated when the HH ligand binds to the transmembrane receptor smoothened (SMO) which is otherwise inhibited by the transmembrane protein patched (PTCH). This results in activation of GLI transcription factors [80].

In RMS, aberrant activation of the HH pathway has been detected in primary tumours due to genetic inactivation of PTCH1, or suppressor of fused (SUFU), or due to amplification of GLI1 [81,82]. Although HH pathway is known to be upregulated in RMS, the HH pathway genes PTCH1, GLI1, GLI3 and Myf5 were expressed to a greater extent in ERMS and fusion-negative ARMS. Interestingly, PTCH1 expression correlated with reduced survival in patients with fusion-negative RMS. Mice heterozygous for patched (PTCH) demonstrated a high incidence for ERMS [83]. In patients with germline mutation in PTCH gene, overexpression of PTCH1 and GLI1 were observed in sporadic RMS that resembled the embryonal subtype [84]. Thus, HH inhibitors may exhibit greater effectiveness in the fusion negative subgroup and also act as a marker for fusion negative RMS tumour aggressiveness [85]. However, some studies have also shown deregulation of HH signalling in ARMS. Inhibition of the HH pathway by forskolin decreased proliferative capacity of both ERMS and ARMS cell lines equally, and in xenograft models, decreased both tumour volumes [86]. High levels of GLI1 mRNA were seen predominantly in ERMS specimens from patients enrolled in Intergroup Rhabdomyosarcoma Study III and IV. However, HH pathway activation did not show any correlation with tumour aggressiveness or other clinical characteristics [87].

Inhibition of the HH pathway with cyclopamine and GANT61 decreased cell proliferation with GANT61 being more effective than cyclopamine. While GANT61 treatment resulted in apoptosis, cyclopamine promoted necrosis. *In vivo* xenograft models also showed reduced tumour growth upon GANT61 treatment [88]. GANT61 also inhibited proliferation in ARMS tumour xenografts and induced apoptosis. Interestingly both HH pathway and mTOR signalling were inhibited by GANT61 along with reduction in epithelial mesenchymal transition (EMT). This study also showed that GANT61 could potentially be used clinically to increase chemosensitivity of RMS cells against mTOR inhibitors rapamycin and temsirolimus as well as the mitotic inhibitor vincristine [89]. Rapamycin was found to inhibit the growth of tumour xenografts of poorly differentiated RMS both by inhibition of the mTOR pathway and HH pathway. The dual inhibition of HH and mTOR by rapamycin also showed a decrease in EMT [90]. In line with this, cyclopamine or forskolin inhibited migratory and invasive capacity of RMS cells [91].

HH pathway also plays a role in self-renewal and tumour initiating capacity in ERMS. NANOG is positively regulated by HH pathway in ERMS. In addition, inhibition of the HH pathway increased chemosensitivity of ERMS cells to irinotecan and doxorubicin. Interestingly, irinotecan increased sphere forming capacity of ERMS that could be rescued by addition of HH pathway inhibitor LDE-225. Thus ERMS containing GLI1 and NANOG seem to have clinically worse outcome [92]. HH pathway was also shown to be induced in cancer stem cell enriched spheres and holoclones of both ERMS and ARMS cell types [93]. IHH, DHH and SHH ligand expression in RMS activates HH signalling in an autocrine manner. Genetic inhibition of IHH and DHH decreased proliferation. However, a cell line with GLI1 amplification was resistant to ligand inhibition due to ligand-independent HH pathway activation. It will therefore be important to determine whether HH activation in patients is ligand-dependent or independent, so that appropriate inhibitors are used [94].

2.7. Hippo signalling pathway

Hippo pathway was first found to be down regulated in fusion positive ARMS. The *PAX3-FOXO1* driven expression of RASSF4 was identified to be responsible for inhibiting the Hippo pathway tumour suppressor MST1 in ARMS to prevent senescence. However, a clear link between RASSF4 and Hippo pathway was not established [95]. A later study found that the YAP oncoprotein which is otherwise inhibited by the Hippo tumour suppressor pathway was higher in ERMS tumours than ARMS tumours. A positive correlation between Ki67, a proliferation marker and YAP1 was evident only in ERMS. YAP1 expression in activated mouse satellite cells gave rise to tumours of the embryonal subtype. This was mediated by YAP1 driving the expression of pro-proliferative genes and oncogenes and repressing MYOD1 and MEF2 differentiation activities to maintain the differentiation block in ERMS. Thus clinically targeting the Hippo pathway may be relevant primarily in fusion negative ERMS patients [96]. RAS mutations are common in ERMS and a subset of RAS-driven tumours are associated with high risk. Hippo pathway plays an important role in RAS driven RMS. A cooperation between YAP and RAs is required for tumour initiation in human cell-based model of RMS. Thus targeting RAS and YAP in combination may prove to be more effective in RAS-driven tumours [97]. Hippo pathway in combination with Notch was also seen to play a role in the cancer stem cell population in ERMS. Interestingly Notch signalling was found to upregulate YAP1 transcription and YAP1 in turn upregulated expression of Notch ligands JAG1 and DLL1 and core Notch transcription factor RBPJ. This positive feedback regulation of both the signalling pathways increased stem cell genes like SOX2. Thus, it will be interesting to determine whether inhibition of either pathway is sufficient to regulate the other and prevent stem cell populations in ERMS, or whether dual inhibition is necessary. While canonical Hippo signalling through YAP seems to play an important role in ERMS, inhibition of Hippo signalling by RASSF4 mediated inhibition of MST1 in *PAX3-FOXO1* ARMS seemed to affect the noncanonical Hippo signalling through MOB1 phosphorylation. In fact, ablation of MST in a genetic ARMS mouse model showed accelerated tumorigenesis [98].

3. Epigenetic alterations

Epigenetic modifications include DNA methylation and histone modifications [99,100]. DNA methylation arises from the transfer of a methyl group from S-adenosyl methionine to DNA, which is catalysed by the DNMT family of enzymes. Histones also play important role in shaping epigenetic landscape. Histones undergo post-transcriptional

modifications such as acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and ADP-ribosylation in the histone tails [101,102]. These modifications are important regulatory mechanisms in transcriptional regulation, DNA repair, alternative splicing, DNA replication and chromosomal compaction. Changes in histone modifications are frequently identified in various cancers due to aberrant expression of histone acetyltransferases and deacetylases (HAT and HDAC). In addition, differential histone methylation marks are found in various cancers. These are caused by altered expression of histone methyltransferases and demethylases. In addition, the physical positioning of nucleosomes is important for regulation of gene transcription. Nucleosome positioning acts as physical hindrance to transcriptional activation when located directly upstream of transcriptional start site. The positions of nucleosomes are regulated by histone variants which can also affect gene expression.

3.1. Histone deacetylases (HDAC)

HDAC inhibitors are effective in inducing apoptosis. For instance, HDAC inhibitors SAHA and pyroxamide resulted in accumulation of cells in sub-G1 phase in RMS cell lines [103]. Two potent HDAC inhibitors vorinostat (SAHA) and panobinostat were found to inhibit tumour growth *in vivo* with induction of apoptosis and inhibition of invasion in cell lines. These effects were due to HDAC-mediated ROS dependent silencing of cMyc that downregulates Specificity transcription factors 1 (Sp1). Sp family of transcription factors are overexpressed in RMS with onco-genic downstream targets. The histone deacetylase function of HDAC inhibitors seemed to have no effect on cancer growth [104]. Trichostatin A (TSA) and SAHA have also been reported to suppress tumour growth primarily by induction of differentiation and inhibition of self-renewal and migratory capacity in ERMS. This effect is seen due to downregulation of Notch1 and EphrinB1 pathways resulting in differentiation and decreased migration in ERMS. Surprisingly, the downregulated Notch1 and Ephrin B1 gene promoters were enriched in acetylated histones indicating that the effects are independent of histone acetylation [105]. HADC inhibitors have also been used clinically. An eight-year old female with ERMS having anaplastic features underwent various conventional therapies and also new experimental ones. However, she faced multiple relapses. The patient was treated with vorinostat based on mutated BCOR, ARIDA and SETD2 genes which are regulated by HDACs. The single agent therapy produced a transient reduction of tumour mass, but was followed by tumour progression. A PDX model developed from the tumour showed induction of necrosis upon vorinostat treatment, but no difference in tumour volume [106]. HDAC inhibitors have also been tested in conjunction with conventional therapies in RMS. SAHA radiosensitized ERMS cells by inhibition of DNA repair and increased apoptosis [107] as well as chemosensitized RMS cells to synergistically increase apoptosis induced by doxorubicin, etoposide, vincristine and cyclophosphamide. The synergy was effective with doxorubicin and etoposide [30]. The effect of HDAC inhibitors in RMS is primarily mediated by induction of ROS, independent of its epigenetic function. However, the effect of entinostat in ARMS was dependent on its inhibition of HDAC3 enzymatic activity. HDAC3 inhibition led to SMARCA4 mediated de-repression of miR-27a which interfered with the stability of PAX3: FOXO1 mRNA. This reduction in *PAX3-FOXO1* by entinostat was able to chemosensitize ARMS cells and tumours to chemotherapy. A phase I clinical trial (ADVL1513) has already shown entinostat to be well tolerated in children, and a phase IB or phase II trial is planned to improve chemosensitivity in ARMS [108].

3.2. Histone acetyltransferases/readers

3.2.1. BET-containing proteins

The role of BET-containing proteins (BRD4,3,2) has only been recently elucidated. in RMS. BET proteins are readers of acetylated lysines on histones. Among the BET proteins, BRD4 showed highest co-localization with *PAX3-FOXO1* at enhancer regions corresponding with H3K27 acetylation marks. BRD4 is essential for *PAX3-FOXO1* function and stability. RMS cells with *PAX3-FOXO1* fusion protein showed selective sensitivity to BET bromodomain inhibition by JQ1 [109]. A recent study investigated combined treatment of the BET inhibitor JQ1 with HDAC inhibitors, JNJ-26481585, SAHA, MS275 or LBH589. All four HDAC inhibitors had synergistic effects with JQ1 in inducing apoptosis with the second-generation JNJ-26481585 being the most potent [110].

3.2.2. P300/CBP-associated factor (P/CAF)

Recruitment of the histone acetyltransferase P/CAF was shown to be necessary for 12-O Tetradecanoylphorbol-13-acetate (TPA) mediated induction of differentiation in ERMS. TPA treatment was found to induce the recruitment of P/CAF on myogenin promoter which correlated with increased acetylation of H3-K9 and H3-K14 and MyoD. This in turn led to increased expression of myogenin [111]. Interestingly, P/CAF binds to *PAX3-FOXO1* fusion protein resulting in its acetylation and stabilisation, which in turn promotes ARMS oncogenesis. P/CAF knockdown or pharmacological inhibition of its acetyltransferase activity by embelin resulted in destabilization of *PAX3-FOXO1* fusion protein. This work provides a potential to therapeutically target the fusion protein in ARMS by targeting chromatin modifying enzymes [112].

3.3. Lysine methyltransferase and de-methylases

3.3.1. K methyltransferase 1A (KMT1A)

KMT1A (SUV39H1) methylates lysine 9 on histone H3 and its expression is elevated in ARMS cell lines. Knockdown of KMT1A in ARMS cell lines activated p21, myogenin, MyoD and MHC resulting in differentiation *in vitro*, and growth inhibition *in vitro* and *in vivo* [113]. Intriguingly, in ERMS, KMT1A has a tumour suppressive role. SUV39H1 overexpressing tumours showed a delay in tumour onset in a KRASG12D driven zebrafish model. The effect of SUV39H1 overexpression was limited to tumour initiation, but once tumours were initiated, it did not affect cell cycle or differentiation [114]. In an effort to find pharmacological inhibitors of KMT1A mediated suppression of differentiation, a drug screen in ARMS picked up a clinically approved topoisomerase 1 inhibitor camptothecin as the strongest hit. Administration of the drug reduced KMT1A levels. Both *in vitro* and mouse models have shown favourable outcomes forming a basis for clinical trials in ARMS [115].

3.3.2. Enhancer of zeste homolog 2 (EZH2)

Polycomb group (PcG) proteins are epigenetic silencers whose expression is de-regulated in a wide range of cancers. EZH2 is the catalytic subunit of the Polycomb Repressive Complex 2 (PRC2) that mediates H3K9me3 marks. EZH2 is overexpressed in both ARMS and ERMS cell lines [116]. Knockdown of EZH2 in an ERMS cell line increased expression of MyoD and its targets, resulting in reduced proliferation and increased differentiation [117]. *In vivo*, 3-Deazaneplanocin A (DZNep), a pharmacological inhibitor of EZH2, or catalytic inhibitors MC1948 and MC1945 that result in EZH2 degradation reduced tumour growth [118]. EZH2 is also elevated in the *PAX3-FOXO1* ARMS and its down regulation by small interfering RNA (siRNA) or pharmacological inhibition using DZNep or MC1945 prevented proliferation. However, the differentiation program was inhibited upon EZH2 inhibition primarily because of increased apoptosis via FBXO32 [119]. Combination of EZH2 inhibitor GSK126 with 2-O-tetradecanoylphorbol-13-acetate (TPA) showed synergistic effects in muscle differentiation in ERMS. TPA is an FDA approved drug for treatment of acute ischemic stroke and inhibits growth and induces differentiation ERMS cells though PKC alpha activation. Designing clinical trials using TPA and EZH2 inhibitors as a combination therapy might be worth considering [120].

3.3.3. Euchromatic histone lysine methyltransferase 1 (EHMT1)/G9a

Recent work from our laboratory has identified the H3K9me2 methyltransferase G9a to be upregulated in ARMS cells and patient samples [121]. Knockdown and pharmacological inhibition of G9a decreased proliferation, migration and invasion as well as removed the differentiation block in ARMS cell lines. This effect was found to be mediated by G9a dependent epigenetic regulation of the PTEN/AKT/RAC1 axis. G9a was found to directly repress the expression of PTEN tumour suppressor in a methyltransferase dependent manner which in turn activated RAC1 and AKT that contributed to the oncogenicity of G9a in ARMS.

3.3.4. Lysine-specific histone demythylase1 (LSD1)

Lysine-specific demethylase 1 (LSD1) has been found to be overexpressed in primary RMS samples. Even though inhibition of LSD1 alone with GSK690 did not have much effect in RMS, combination with HDAC inhibitor JNJ-26481585, synergistically induced cell death [122].

3.4. DNA methyltransferases

There are three DNMTs in mammals: DNMT1, 3 A and 3B. DNA methylation has been shown to play important role in different cancers, primarily by mediating silencing of tumour suppressor genes. RMS have been characterized by distinct DNA methylation patterns with hypermethylated CpG islands in genes involved in skeletal muscle development and differentiation. Indeed, treatment with 5-aza-2′-deoxycytidine (5-aza-dC), a DNA de-methylating agent decreases proliferation and migration, and also increased differentiation through modulation of microRNAs mir-378 and miR-675 [123]. DNMT1 and DNMT3B are highly expressed in both ERMS and ARMS. DNMT3B knockdown led to decreased proliferation and migration and increased differentiation in ERMS cells [124]. Interestingly, 5-aza-dC and valproic acid significantly reduced RMS tumour development in PTCH heterozygous mice that spontaneously develop either medulloblastoma or rhabdomyosarcoma, while no difference in tumour growth was observed once the tumours were formed. The treated mice showed an upregulation of wild type PTCH expression [125].

4. Oxidative stress

ROS levels are elevated in RMS due to de-regulation of MCU/MICU1 expression and several differentially methylated genes like PTK2, COX7A1, NOSIP, NOS1, ATP2A3, DDAH1, GLRX and TXNDC12 which play important roles in metabolism, mitochondrial dysfunction and oxidative stress [126]. Elevated levels of G→T transversions, considered to be oxidative damage mediated mutations, are also elevated in ERMS compared to other paediatric tumours. High throughput screening of primary cultures from ERMS orthotopic tumour xenografts showed agents targeting oxidative stress to have the most effective response. Most HDAC inhibitors showed activity against ERMS xenografts, with panobinostat showing highest activity. Other ROS producing compounds like carfilzomib, auranofin, cerivastatin, alvocidib and ouabain also were active against ERMS xenografts. All of these drugs increase ROS production in RMS.

This may explain the susceptibility of this cancer to treatments that further increase oxidative stress. For instance, actinomycin-D, one of the most successful treatments against RMS, induces oxidative stress [127,128]. Another strategy to increase oxidative stress is by targeting detoxifying enzymes like thioredoxin (TXN) reductase a key enzyme responsible for neutralization of ROS. Indeed treatment of ERMS xenografts with auranofin, a TXN reductase inhibitor, along with HDAC inhibitor panobinostat reduced tumour growth [129]. HDAC inhibitors panobinostat and vorinostat are known to inhibit tumour growth and induce apoptosis in RMS primarily through their induction of ROS which is independent of histone acetylation [104]. TXN reductase is constantly regenerated by NADPH, which is generated by the pentose phosphate pathway, cytosolic isocitrate dehydrogenase and NADP-dependent malic enzymes. Glucose-6-phosphate dehydrogenase enzyme is known to regulate the first step of pentose phosphate pathway. Thus one mechanism by which glucose-6-phosphate dehydrogenase silencing in ERMS impairs proliferation might be through induction of ROS [130]. Artesunate, a semi-synthetic derivative of artemisin resulted in induction of apoptosis upon DNA damage and ROS induction in ERMS. Activation of ROS in ERMS increases expression of myo-miRs, miR-133a and miR-206 that in turn reduce *PAX7* [131]. Induction of ROS was also seen when RMS cells were treated with the fungicide ciclopirox olamine that caused autophagy through JNK pathway [132]. However recent studies have also shown that treatment of ERMS cells with mitochondria targeted antioxidant SkQ1 which can scavenge ROS mitochondrial ROS resulted in growth suppression by prolonging mitosis and apoptosis induction [133]. While RMS is known to have high ROS levels, it is important to note that cancer stem cells (CSC) in many cases have lower levels of ROS [134,135] likely due lower level of ROS production or increased signalling pathways and transcriptional activity that can scavenge ROS. Notch pathway is implicated in cancer stem cell maintenance and expansion in various cancers [136] including RMS [45,137]. Notch pathway, which activates AKT pathway in glioma stem cells [138] might be responsible for upregulating ROS scavenging enzymes [139] and thereby for maintenance of low ROS levels in cancer stem cells. Conversely, ROS stimulates Notch signalling. For instance, nitric oxide from endothelial cells promotes stem cell characteristics of PDGF-induced glioma cells by activating Notch signalling [140].

RAS GTPase family has been shown to be closely associated with ROS production and can either act as upstream regulator or downstream effector of ROS [141,142]. Since ROS is highly regulated in skeletal muscle cells through a robust antioxidant defence system, any de-regulation in ROS due to disrupted RAS signalling can be oncogenic. Elevated RAS signalling can be one of the main drivers for excess ROS in RMS tumour cells [73]. The elevation of ROS is important to drive RAS dependent cell proliferation in promoting tumour progression [142]. Hence, targeting RAS mutation and oxidative stress can be potential therapeutic avenue for RMS.

There are various therapies in clinical trials that work by inducing ROS levels. Procarbazine was one of the first ROS inducing agents to be used in cancer treatment. Following successful clinical trials, procarbazine was approved for treatment of Hodgkin's lymphoma, non-Hodgkin's lymphoma and primary brain tumours [143]. Another ROS inducing drug approved for the treatment of non-Hodgkin's lymphoma is rituximab, an anti-CD20 monoclonal antibody that induces ROS production in lymphoma cells [143]. Phase I and II clinical studies have shown the use of another ROS inducing arsenic agent, imexon to be safe for the treatment of leukaemia and other cancer types [144]. Clinical trials have also been carried out with drugs targeting the cellular antioxidant systems that leads to increased ROS mediated cytotoxicity in cancer cells. Motexafin gadolinium is a thioredoxin inhibitor that causes tumour cell specific cytotoxicity. It has undergone Phase I clinical trial along with docetaxel and cisplatin in patients with lung cancer. The drug was found to be tolerable with some favourable activity in patients with metastatic non-small cell lung cancer. Methoxyestradiol (2-ME), a superoxide dismutase (SOD) inhibitor, is another antioxidant enzyme inhibitor that has been tested in patients with solid tumours in a phase I clinical trial. Despite no dose limiting toxicities, the trial was suspended due to very low plasma concentration of the drug [145]. A phase II study with 2-ME2 was carried out in multiple myeloma patients. Minor responses and prolonged plateau phase diseases was observed in some patients. The result seemed promising but needs to be developed into a more bioavailable formulation [146]. A list of clinically approved drugs used in other cancers that has been shown to induce ROS production in preclinical RMS models can be found in Table 2. [147–154]

5. Conclusions and future directions

A molecular understanding of the genetic and epigenetic landscape and the altered redox imbalance provides new targets for therapeutic intervention in RMS. In particular, the highly specific enzymatic activities of chromatin modifiers have been explored from a therapeutic standpoint. Despite promising results in preclinical studies however, the efficacy of pharmacological inhibition in the clinic needs to be demonstrated and the long-term impact remains to be investigated. A case in point is a recent study where epidermal deletion of G9a resulted in slower tumour initiation upon carcinogen insult, but was followed by development of more aggressive and metastatic tumours with increased genomic instability in the long-term [155]. Thus, caution needs to be exercised and the effects of epigenetic drugs, especially when used in combination therapy, should be tested for mutation profiles and genome stability. Nonetheless, the identification of novel oncogenic epigenetic proteins and regulatory circuits will result in new drug targets to which small molecule inhibitors can be screened and designed for use as standalone or combinatorial/adjuvant therapy. Some epigenetic drugs that have been shown to have inhibitory effect in ARMS and ERMS tumour growth in preclinical and clinical trials are listed in Fig. 3. Epigenetic regulators can also be used to define distinct subsets of RMS patients based on their expression level and thus be used as novel diagnostic/prognostic markers and allow for improved personalized treatment strategies. Finally, defining the regulatory cross talk between genetic/epigenetic alterations and the redox imbalance in RMS (Fig. 4) will likely lead to development of targeted therapies for therapy recalcitrant tumours.

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