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Merlin: The wizard requires protein stability to function as a tumor suppressor:

Is stability important for tumor suppressor activity?

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Abstract

Neurofibromatosis type 2 (NF2), characterized by tumors of the nervous system, is a result of functional loss of the *NF2* gene. The *NF2* gene encodes Merlin (moesin-ezrin-radixin-like protein), an ERM (Ezrin, Radixin, Moesin) protein family member. Merlin functions as a tumor suppressor through impacting mechanisms related to proliferation, apoptosis, survival, motility, adhesion, and invasion. Several studies have summarized the tumor intrinsic mutations in Merlin. Given the fact that tumor cells are not in isolation, but rather in an intricate, mutually sustaining synergy with their surrounding stroma, the dialogue between the tumor cells and the stroma can potentially impact the molecular homeostasis and promote evolution of the malignant phenotype. This review summarizes the epigenetic modifications, transcript stability, and post-translational modifications that impact Merlin. We have reviewed the role of extrinsic factors originating from the tumor milieu that influence the availability of Merlin inside the cell. Information regarding Merlin regulation could lead to novel therapeutics by stabilizing Merlin protein in tumors that have reduced Merlin protein expression without displaying any *NF2* genetic alterations.

Keywords

Merlin; NF2; neurofibromatosis; stability; tumor microenvironment

Introduction

Neurofibromatosis type 2 (NF2) is an autosomal dominant disease affecting 1 in 30,000 children and young adults [1]. This disease is caused by a loss of heterozygosity of the *NF2* (neurofibromatosis-2) gene located on chromosome 22q12 [2]. *NF2* is a classical tumor suppressor gene that is frequently inactivated or its expression lost as a result of mutations in tumors of the nervous system such as schwannomas, meningiomas, and ependymomas [3-7]. While infrequent, loss of *NF2* function due to mutations has been documented in other non-nervous system cancers such as mesotheliomas [8-10], colorectal cancer [11], prostate

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cancer [12], melanoma and thyroid cancer [13]. The presence of mutations in non-nervous system tumors accompanied by loss of *NF2* function suggests that this tumor suppressor functions in a broad range of tissue types.

Merlin, also known as schwannomin or neurofibromin 2 was first discovered in 1993 as the protein encoded by the *NF2* gene [14, 15]. Merlin is a member of the Band 4.1 family of cytoskeletal linker proteins that include the ERM (Ezrin, Radixin, Moesin) proteins [16]. Traditionally, proteins of this family function to process signals from the extracellular matrix and transmit these signals downstream to proteins inside the cell. Loss of Merlin function lends cells of the nervous system to unchecked proliferation and motility which results in the formation of the non-malignant tumors that NF2 patients present. Loss of Merlin is embryonic lethal in mice, indicating that Merlin is a critical molecule expressed during normal embryonic development [17, 18]. Moreover, heterozygous Merlin knockout mice (*NF2*^{+/-}) develop a series of highly metastatic tumors including hepatocellular carcinomas, fibrosarcomas, and osteosarcomas [19]. Cumulatively, this suggests that Merlin may function to inhibit tumor growth and progression in a variety of cell types. This review will focus on Merlin's tumor suppressor function with a large portion devoted to the various mechanisms by which Merlin is regulated.

Merlin Functions as a Tumor Suppressor

Merlin has been shown to inhibit tumor growth and suppress malignant activity of cancer cells through multiple mechanisms. There are three main mechanisms by which Merlin acts to inhibit tumor growth: Contact-dependent growth inhibition, decreased proliferation, and increased apoptosis. Merlin initially was discovered to reverse the Ras-induced phenotype and restore contact-inhibition of growth [20]. Morrison *et al* showed that Merlin disrupts the Ras and Rac signaling pathways leading to contact-dependent growth inhibition [21]. Evidence suggests that Merlin can suppress Ras-induced transformation through several mechanisms including binding to RalGDS (Ral guanine nucleotide dissociation stimulator) [22], binding to and inhibiting p21-activated kinase [23, 24], and inhibiting Rac/Cdc42 [25]. More recently, Yi *et al* demonstrated that Merlin complexes with the tight junction-associated protein, Angiomotin, and functions to suppress cell growth by inhibiting Rac1 and Ras-MAPK signaling [26].

Several studies have revealed the ability of Merlin to negatively regulate cell growth and proliferation [27-29]. Kim *et al* showed that Merlin can induce apoptosis upon over-expression, in part by causing degradation of Mdm2 leading to the increased stability and overall tumor suppressor function of p53 [30]. Others have shown that Merlin can inhibit cell cycle progression through suppression of PAK1-mediated expression of Cyclin D1 [31]. Merlin's function as a tumor suppressor by negative regulation of cell proliferation and induction of apoptosis is conserved in *Drosophila* [32]. Merlin can also reduce cell proliferation by binding to the cytoplasmic tail of the CD44 receptor. This binding inhibits the interaction of Hyaluronic Acid (HA) with CD44 and suppresses downstream signaling events [28, 33]. More recent studies have focused on previously undefined roles for Merlin such as nuclear translocation to inhibit the E3 ubiquitin ligase CRL4 (DCAF1) resulting in decreased proliferation in schwannomas [34, 35]. In addition, Merlin has been shown to sequester EGFR in mouse embryonic fibroblasts (MEFs) and halt downstream signaling [36, 37] resulting in decreased cell proliferation. Based on this information, a clinical trial was developed to study the effect of erlotinib (a small molecule inhibitor of EGFR) in patients with progressive vestibular schwannoma. The study concluded that there was no tumor response using this inhibitor alone suggesting that Merlin's tumor suppressor effect is not mediated solely through EGFR [38]. James *et al* showed that constitutively active mammalian target of rapamycin complex 1 (mTORC1) in Merlin-deficient meningioma

cells led to increased cell growth [39]. NF2 patient tumors as well as *Nf2*-deficient MEFs displayed elevated mTORC1 signaling. Re-introduction of Merlin suppressed mTORC1 signaling. This group also found that Merlin inhibits mTOR signaling through a novel PI3-Kinase/AKT-independent mechanism which may lead to combination therapies including rapamycin or other mTOR inhibitors. Although Merlin has been shown to inhibit several signaling pathways that are important for tumor growth and progression, it is likely that future studies will reveal many more aspects of Merlin activity. Merlin regulation studies have largely been focused on mutational events. However, there are other important regulatory mechanisms determining Merlin's availability in a given cell. These include epigenetic alterations, transcript stability, and post-translational modifications. There is a significant body of literature that details the various mutations associated with loss of Merlin [40, 41]. This review focuses on aspects of Merlin regulation that include epigenetics, transcript stability and post-translational modifications that are influenced by the dialogue between the tumor cells and their microenvironment.

Epigenetic Modifications

Although mutations in the *NF2* gene cause tumors of the nervous system, there are likely multiple mechanisms that account for the inactivation of the Merlin protein. Promoter methylation has been shown to cause silencing of several tumor suppressor genes including *E-cadherin* [42], *p16* [43], and *VHL* [44]. Although there are likely several reasons for Merlin inactivation, promoter methylation is the only epigenetic modification that has been associated with changes in Merlin expression. Thus far there is no available literature on promoter deacetylation with regard to regulation of Merlin. Kino and colleagues found that nearly 60% of tumors from schwannoma patients displayed methylation of the *NF2* promoter at three different sites within a CpG island. They also noted that Merlin mRNA expression was consistent with the methylation status [45]. Another study confirmed *NF2* promoter methylation as a frequent event in schwannomas although at a much lower rate [46]. More recently, it was determined that there were a significant number of sporadic vestibular schwannoma patients that did not exhibit methylation of wild-type *NF2* (>40%) [47]. Given the discrepancy regarding *NF2* methylation in schwannomas, several studies aimed to determine the methylation status in other tumors of the nervous system such as meningiomas and ependymomas. One study confirmed that *NF2* methylation was a rare event (1 of 21) in meningioma patients [48], with another study only detecting methylation at one CpG site in 1 out of 12 tumor samples [49]. An independent study determined that *NF2* methylation occurred in less than 10% of ependymoma cases analyzed [50]. It appears that promoter methylation may be important for *NF2* regulation, but further investigation is required to confidently make this assertion.

mRNA Stability

Transcript stability is another important aspect that controls tumor suppressor protein expression. Tumor suppressors such as p53 [51] and p21 [52] are known to be regulated at the mRNA level by other proteins and miRNAs. Evidence is inconclusive as to whether Merlin mRNA stability plays a role in tumor progression and also which mechanisms (miRNA signaling or mutational events) are important for Merlin transcript stability. Since there are no studies yet concerning regulation of Merlin mRNA by miRNAs, this section will be centered on whether mutational events are necessary for stability of Merlin mRNA. Hoang-Xuan *et al* found that Merlin transcript levels are not altered in gliomas [53], while Jacoby *et al* determined that different mutations resulted in varying degrees of Merlin mRNA expression in NF2 and schwannomatosis [54]. Another study in meningiomas showed that tumors from patients harboring *NF2* mutations had 10-fold lower Merlin transcript levels [55]. Deguen *et al.* showed that 11 of 18 human malignant mesothelioma

(HMM) cell lines exhibited decreased Merlin transcript levels, 4 of which displayed no detectable mutations [56]. With the limited available literature focusing on Merlin transcript stability, it appears that a reduction in Merlin mRNA levels is associated with mutations of the *NF2* gene. Moreover, in the absence of mutations, Merlin transcript stability is unaltered. Three independent studies confirmed the absence of mutations in the *NF2* gene in breast cancer [57-59]. Kanai *et al* reported no mutations of *NF2* upon analysis of 68 patient samples, Arakawa *et al* detected no mutations in samples from 55 patient tissues, and Yaegashi *et al* observed a mutation in only 1 out of 60 patient tissues [57-59]. Cumulatively, these studies suggested that mutation of the *NF2* gene is an infrequent event in breast cancer. Our recent study confirmed a lack of change in Merlin transcript levels in advanced breast cancer compared with normal breast tissues [60]. Although there are correlative results concerning Merlin transcript stability, more investigation into this area of Merlin regulation is needed.

Post-translational Regulation of Merlin

The ERM proteins function as molecular linkers by connecting the cytoskeleton with the plasma membrane [61]. Although the ERM proteins and other related proteins share a central α -helical domain, Merlin lacks an actin-binding motif that is present at the carboxy-terminus in other ERM proteins [62, 63]. This suggests that Merlin may have functions distinct from other ERM family members. Merlin is able to form homodimers with itself and also heterodimers with other ERM protein family members via head-to-tail intra- and inter-molecular associations [64-66] (Figure 1). Merlin's tumor suppressive activity is dependent on its ability to form a head-to-tail association resulting in a closed, active conformation [27] (Figure 1). A recent crystallographic study suggested that the "closed" conformation of Merlin is in fact an "open" dimer of two Merlin molecules and that this association is responsible for Merlin's tumor suppressor activity [67]. Myosin phosphatase MYPT1-PP1 δ dephosphorylates Merlin at Ser518 in response to high cell density or serum withdrawal and thereby activates Merlin [28, 68] (Figure 1). However, the oncogene protein kinase C-potentiated phosphatase inhibitor of 17 kDa (CPI-17) has been shown to inhibit Merlin activity in mesothelioma by inhibiting the targeting subunit of MYPT1-PP1 δ [68, 69] (Table 1).

Merlin acts as a mediator of extracellular signals to intracellular proteins. In the context of its microenvironment, the tumor cells are recipients of a multitude of extracellular cyto/chemokines, all of which profoundly impact the tumor cell behavior. The reactive stroma surrounding the tumor engages in a two-way dialogue with the tumor cells. The tumor milieu comprises infiltrating immune cells, complement proteins, endothelial cells, pericytes and growth inhibitory as well as growth promoting cytokines. Inflammatory conditions in the tumor microenvironment promote proliferation and survival of malignant cells, angiogenesis, metastasis, subversion of adaptive immunity, response to hormones and chemotherapeutic agents. This tumor microenvironment is also rich in cytokines and growth factors that can enhance many signaling pathways.

In response to growth factors, low confluence in culture, or cell/substrate attachment, Merlin is phosphorylated at Ser518 by either cAMP-dependent protein kinase (PKA) or p21-activated kinase 1/2 (PAK1/2). This phosphorylation results in an open, inactive conformation and loss of tumor-suppressor activity of Merlin [27, 70-73] (Figure 1, Table 1). *NF2* missense mutations in either the N-terminus or C-terminus maintain Merlin in an "open" conformation, one that does not suppress cell growth [74]. In response to growth factors such as EGF (epidermal growth factor) or IGF (insulin growth factor) that are abundant in the tumor milieu, AKT phosphorylates Merlin at Thr230 and Ser315. This phosphorylation targets Merlin for proteasomal degradation by ubiquitination [75] (Table 1).

A recent study revealed Ser10 as a novel AKT phosphorylation site that targets Merlin for proteasomal degradation [76] (Table 1).

We recently demonstrated in breast cancer that the availability of Merlin protein (assessed by immunohistochemistry and subsequent scoring) was compromised despite no significant changes at the transcript level. In breast cancer Merlin protein is targeted for degradation by AKT-mediated phosphorylation at Ser315 in response to the tumor microenvironment-associated cytokine, osteopontin. Our study demonstrated that breast tissues displaying loss of Merlin expression show a concomitant increase in osteopontin expression as determined by immunohistochemistry in 77% (58 of 75) of patient tissues analyzed [60]. Osteopontin is a secreted positive regulator of tumor progression and metastasis [77, 78] that is readily available in the tumor microenvironment and elevated in multiple cancer types including advanced breast cancer [79-82]. Osteopontin promotes pro-inflammatory conditions of the tumor milieu [83] and acts as an effector of tumor progression and metastasis at several levels. Osteopontin signals via multiple receptors including several integrins and CD44, activates NF- κ B, PI-3-kinase and AKT signaling and enhances malignant attributes [77].

While osteopontin induced ubiquitin-mediated degradation of endogenous (wild-type) Merlin in the breast cancer cells, an exciting aspect of this study was that a degradation-resistant Merlin mutant was able to suppress malignant activity of breast cancer cells even in the presence of osteopontin. This suggests a novel mechanism of Merlin regulation, as well as an opportunity for therapeutic intervention in tumors that display elevated levels of osteopontin or other growth factors such as EGF, either in the tumor cells themselves or in the extracellular milieu. Thus, Merlin is modulated by factors extrinsic to the tumor itself, necessitating the need to assess the Merlin at the protein level rather than simply evaluating tumor-intrinsic genetic mutations in Merlin. More work is necessary to determine the full implications of post-translation modifications of Merlin protein and when these modifications are important for disease.

Discussion

The vast majority of literature concerning Merlin focuses on mutational events within the *NF2* gene, resulting in a non-functional Merlin protein. Given that the topic of mutational events has been widely discussed, this review has concentrated on the stability of Merlin protein in the absence of mutational events. At this point, it is unclear whether Merlin protein can be regulated by epigenetic modifications such as promoter methylation. There have been relatively few studies aimed at uncovering the role of Merlin mRNA stability in tumor progression. It appears that further efforts are warranted since there seems to be a correlation between mutational events and overall transcript levels. However, there is little evidence that Merlin mRNA stability plays a role in tumor progression in the absence of deleterious mutations. Despite the fact that *NF2*/Merlin loss has been studied extensively at the genomic level, there have been relatively few studies focusing on post-translational modifications. These studies will be important in malignancies like breast cancer where no mutational events are detected. A loss of Merlin protein expression may still occur without any change at the transcript level [60]. More work is necessary in determining the exact pathways that lead to phosphorylation which seems to inhibit Merlin tumor suppressor activity. Further investigation into mechanisms of dephosphorylation is also warranted. The lone Merlin phosphatase, MYPT1-PP1 δ , has been established as a positive regulator of Merlin tumor suppressor activity by relegating Merlin to its closed, active conformation. However, there is little knowledge about its expression and activity levels in cancers negative for Merlin mutations. To date, no phosphatase(s) have been identified that regulate Merlin stability. The identification of these potential phosphatases could lead to the development of therapies aimed at stabilizing Merlin protein.

Merlin is not the only tumor suppressor that is regulated at the level of protein stability. Degradation of the p53 and Rb tumor suppressor genes by Mdm2 have been well documented to contribute to tumorigenesis [84]. In breast cancer, it has recently been demonstrated that the nucleolar protein ZNF668 (zinc finger protein 668) can inhibit proliferation *in vitro* and tumorigenicity *in vivo* by increasing the stability of p53 [85]. Moreover, in lung cancer a recent report showed that the anti-apoptotic protein TCTP (translationally controlled tumor protein) promotes the degradation of p53 protein [86] which leads to reduced apoptosis. Inhibition of TCTP leads to stabilized p53 and increased apoptotic activity. Furthermore, the E3 ubiquitin ligase Itch was found to negatively regulate the large tumor suppressor (LATS1) by targeting LATS1 for polyubiquitination and proteasomal degradation [87]. Upon knockdown of Itch, endogenous LATS1 proteins were stabilized, suggesting that Itch expression levels are important for the post-translational regulation of the LATS1 tumor suppressor. In breast cancer, the tumor suppressor PTEN (phosphatase and tensin homolog) is frequently downregulated at the protein level in the absence of promoter methylation or genetic alterations [88-90]. Yim *et al* demonstrated that enhanced binding of E3 ubiquitin ligase leads to ubiquitination and degradation of PTEN [91]. Conversely, evidence suggests that PICT-1 (protein interacting with carboxyl terminus 1) physically interacts with PTEN, promotes C-terminal phosphorylation, and enhances PTEN protein stability in MCF7 breast cancer cells [92]. Further, protein kinase CK2 was shown to phosphorylate PTEN on C-terminal serine/threonine residues leading to increased protein stability [93]. Though the importance of tumor suppressor stability regulation is becoming clearer, the extracellular signals that dictate this stability have yet to be elucidated. However, our laboratory recently found in breast cancer that the growth and metastasis-promoting inflammatory cytokine, osteopontin, signals the degradation of the tumor suppressor Merlin mediated by AKT phosphorylation [60]. Moreover, it is important to note that osteopontin is readily available in the tumor microenvironment. The expression of osteopontin is increased in advanced grade breast tumors. Our study demonstrated that low Merlin expression correlated with high osteopontin expression as determined by immunohistochemistry in 77% (58 of 75) of patient tissues. This provides evidence that the tumor microenvironment can play a critical role in post-translational stability of tumor suppressor proteins.

Given this information, it seems logical that proteasome inhibition might serve as a critical therapeutic strategy. Indeed, there have been numerous clinical trials conducted using the proteasome inhibitor bortezomib as a single agent in non-small cell lung cancer [94], androgen-independent prostate carcinoma [95], as well as multiple myeloma and non-Hodgkin's lymphoma [96]. Bortezomib has been approved for use in certain subsets of patients with relapsed/refractory myeloma after data showed bortezomib induced a better overall response rate than the previous standard dexamethasone [97, 98]. In cases where chemoresistance is evident, bortezomib has been combined with several chemotherapy agents including docetaxel [99], pegylated liposomal doxorubicin [100], and carboplatin [101] among others. In metastatic breast cancer bortezomib has been combined with pegylated liposomal doxorubicin in a phase II clinical trial showing minimal activity [102]. A separate phase II trial was conducted in patients with endocrine-resistant metastatic breast cancer combining anti-hormonal treatments with bortezomib. This study similarly showed no objective antitumor response [103] suggesting that proteasome inhibition is not sufficient for treatment of advanced breast cancer even when combined with other chemotherapeutic agents.

Given the fact that tumor cells are not in isolation, but rather in an intricate, mutually sustaining synergy with their surrounding stroma, the dialogue between the tumor cells and the stroma can potentially impact the molecular homeostasis and promote evolution of the malignant phenotype. This highlights the need for additional methods of Merlin

stabilization, in tumors showing no mutations in *NF2* gene, such as inhibition of the activity of tumor-promoting cytokines (or their receptors) that are readily available in the tumor microenvironment.

While the majority of information regarding loss of Merlin function has been centered on mutational events leading to non-functional Merlin protein, this review has focused on stability of Merlin protein. Post-translational modifications such as phosphorylation have been documented to regulate Merlin stability in the absence of mutations. Our recent report demonstrated that a tumor microenvironment-associated cytokine, osteopontin, targets Merlin for degradation mediated by AKT phosphorylation in breast cancer. If we can target tumor-associated molecules such as cytokines and growth factors or their receptors, we could stabilize tumor suppressor proteins like Merlin within the cell. This approach may lead to tumor reduction in tissues that display no mutational events in the *NF2* gene. In tumors that have lost Merlin expression, the AKT pathway activation is increased [104, 105]. This provides another avenue for therapeutic intervention. There is a significant amount of research underway to target AKT. There are several clinical trials in all different phases currently aimed at targeting the AKT pathway in multiple cancer types including lymphoma, head and neck cancer, melanoma, and breast cancer. While it is still early, targeting the AKT pathway shows promise as the inhibitor triciribine was well-tolerated and showed pharmacodynamic activity as measured by decreased active AKT in a phase I trial in patients with advanced hematologic malignancies, including refractory/relapsed acute myelogenous leukemia [106]. This inhibitor can also suppress growth in breast cancer cell lines that have endogenously high levels of AKT, but not in tumors that contain low levels of AKT [107]. Perifosine is an oral AKT inhibitor that inhibits cell growth concomitant with an increase in apoptosis in breast cancer models [108]. Given this information, it appears that drugs aimed at downstream effector molecules hold significant promise in malignancies that lack active tumor suppressor proteins. In addition, drugs aimed at inhibiting activity of tumor-associated cytokines or their receptors may result in stabilization of tumor suppressor proteins like Merlin, possibly leading to tumor reduction. This strategy holds enormous potential as the role of stabilizing tumor suppressor proteins in patients has been vastly understudied.

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Abbreviations

AKT	Protein kinase B
Cdc42	Cell division control protein 42 homolog
CpG	Cytosine-phosphate-guanine
CPI-17	C-potentiated phosphatase inhibitor of 17 kDa
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERM	Ezrin, Radixin, Moesin
HA	Hyaluronic acid
HMM	Human malignant mesothelioma
IGF	Insulin growth factor
LATS1	Large tumor suppressor 1
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
miRNA	micro RNA
mRNA	messenger RNA
MYPT1-PP1δ	Myosin phosphatase targeting subunit1-protein phosphatase1 delta
NF2	Neurofibromatosis type 2
NF2	Neurofibromin 2
PAK	p21-activated kinase
PICT-1	Protein interacting with carboxyl terminus 1
PKA	cAMP-dependent protein kinase
PTEN	Phosphatase and tensin homolog
RalGDS	Ral guanine nucleotide dissociation stimulator
RNA	Ribonucleic acid
Ser	Serine
TCTP	Translationally controlled tumor protein
Thr	Threonine
VHL	Von-Hippel Lindau
ZNF668	Zinc finger protein 668

Figure 1. Schematic representation of the effect of Merlin activity/stability on tumorigenesis
 Shown in the schematic is the effect of the growth-promoting cytokines (EGF, IGF, osteopontin etc.) that are either produced by the tumor or are available to the tumor as a component of its microenvironment. The mitogenic response-stimulated phosphorylation of Merlin protein by PKA/PAK/AKT causes Merlin to switch to open, inactive conformation upon its phosphorylation at Serine 518. Phosphorylation of Merlin at Threonine 230/Serine 315 causes Merlin to be marked for proteasomal degradation, leading to the loss of availability of Merlin to function as a tumor suppressor, promoting subsequent malignant progression. MYPT1-PP1δ dephosphorylates Merlin allowing Merlin to fold back into closed, active conformation. This conformation permits Merlin to perform its tumor suppressor function. (CAF represents cancer associated fibroblasts)

Table 1

Known phosphorylation sites and their effect on Merlin activity/stability.

Phosphorylation Site	Kinase/Phosphatase	Result	Functional Effect	References
Serine 10	PKA/AKT	May target Merlin for degradation	Altered cell morphology, growth permissive	Laulajainen <i>et al.</i>
Threonine230/Serine 315	PAK1/2, AKT	Targets Merlin for proteasomal degradation	Growth permissive	Ye <i>et al.</i> , Tang <i>et al.</i> , Morrow <i>et al.</i>
Serine 518	PKA, PAK1 Myosin phosphatase (MYPT1-PP1δ)	Switches Merlin to open conformation	Growth permissive	Sherman <i>et al.</i> , Morrison <i>et al.</i> , Kissil <i>et al.</i> , Xiao <i>et al.</i> , Rong <i>et al.</i> , Thaxton <i>et al.</i> , Jin <i>et al.</i> , Thurneysen <i>et al.</i>