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The TRE17/USP6 Oncogene: a riddle wrapped in a mystery inside an enigma

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TABLE OF CONTENTS

1. ABSTRACT

De-ubiquitinating enzymes (DUBs) play critical roles in diverse cellular processes, including intracellular trafficking, protein turnover, inflammatory signaling, and cell transformation. The first DUB to be identified as an oncogene was TRE17/Ubiquitin-specific protease 6 (USP6)/Tre-2. In addition to encoding a USP, TRE17 also contains a TBC (Tre-2/Bub2/Cdc16) domain implicated in GTPase regulation and trafficking. Though first described almost two decades ago, remarkably little has been elucidated regarding TRE17's molecular and cellular functions. However, recent work has implicated TRE17 as a key etiological factor in aneurysmal bone cyst (ABC), a locally recurrent pediatric bone tumor, and identified potential pathways through which it acts. In this review, we discuss the most up-to-date findings on the molecular functions of TRE17, the role of its USP and TBC domains, and potential models for how it contributes to transformation and ABC pathogenesis.

2. INTRODUCTION

The modification of proteins by ubiquitination plays a central role in cellular homeostasis, regulating processes as diverse as protein turnover, vesicular trafficking, inflammatory signaling, gene regulation, and the DNA damage response (for several excellent reviews, see (1-3)). De-ubiquitinating enzymes (DUBs), which catalyze the removal and processing of ubiquitin chains from modified substrates, constitute a large family of almost 100 members in humans. Among the five sub-families of DUBs, the ubiquitin-specific proteases (USPs) constitute the most populous group, with over 50 predicted members. USPs typically contain motifs dedicated to substrate binding and regulation of enzymatic activity, and a major goal in the ubiquitin field is unraveling the specificity, regulation, and cellular functions of this diverse family (1-3). One of the first USPs to be characterized was TRE17/USP6/Tre-2 (4).

In addition to its USP domain, TRE17/USP6/Tre-2 also possesses a TBC domain, which is predicted to function as a regulator of small GTPases, specifically of the Rab subfamily. Rabs constitute the largest subfamily of GTPases, and assume central roles in all aspects of vesicular transport (5). Cycling between the active GTP-bound and inactive GDP-bound state is essential for their function in trafficking. Two classes of proteins regulate this conversion: GTPase-activating proteins (GAPs) inactivate G proteins by stimulating their intrinsic GTPase activity, and guanine nucleotide exchange factors (GEFs) induce activation by catalyzing loading of GTP. GAPs for Rabs are encoded by TBC (Tre-2/Bub2/Cdc16) domains. Over 50 TBC domain-containing proteins are predicted to exist in humans, and as with USPs, a key issue in the field is dissecting their specificity and regulation (6). Although TRE17/USP6/Tre-2 is one of the namesakes of this domain, it has turned out to be an atypical TBC protein, as it neither functions as a GAP, nor targets a Rab (7-9).

While TRE17 was one of the first members of both the USP and TBC families to be described (4), our understanding of its molecular, cellular, and organismal functions remains in its infancy. An important advance came in 2004, when TRE17 was found to possess a direct role in human neoplasia, specifically in aneurysmal bone cyst (ABC), a bone tumor of otherwise unknown etiology (10, 11). Since this discovery, little progress has been made in understanding how TRE17 contributes to the pathogenesis of this tumor. However, the recent establishment of cellular systems to analyze its functions has begun to shed light on the role of this elusive protein in human neoplasia.

In this review, a brief history of TRE17/USP6/Tre-2 will first be provided (from its initial identification, to its evolutionary origin, to its involvement in ABC). This will be followed by a summary of its functional domains, binding partners, and the cellular pathways it engages. In the final sections, we will speculate how these different pathways contribute to ABC pathogenesis, consider potential novel therapeutic approaches, and discuss the key issues that remain for understanding TRE17's role in human neoplasia.

3. TRE17/USP6 BACKGROUND

3.1. Identification and expression of TRE17

TRE17/USP6/Tre-2 (hereafter referred to as TRE17) was first identified as a novel proto-oncogene in 1992 (4). It was cloned by introducing genomic DNA from the IARC-EW1 Ewing sarcoma cell line into NIH3T3 fibroblasts, then screening for transformants that caused tumor formation in nude mice (4, 12). Northern blot analysis suggested that *TRE17* was highly expressed in IARC-EW1 cells, as well as a number of other cancer cell lines. However, subsequent studies indicated that these positive Northern signals likely derived at least in part from cross-reactivity with the *USP32* gene, which is widely expressed and whose messenger RNAs are identical in size to those of *TRE17* (13). More recent studies based on reverse transcription polymerase chain reaction (RT-PCR) using primers that selectively amplify *TRE17* indicate that its expression in both normal tissues and primary tumors is restricted (13-15). In one study, high TRE17 expression was disproportionately observed in tumors of mesenchymal origin (4 out of 29 tumors), but was absent from tumors of epithelial origin (0 out of 22 tumors), despite the fact that the latter constitute the overwhelming majority of cancers. This was not due to higher expression in mesenchymal lineages in general, since normal mesenchymal cells/tissues did not express TRE17 (4, 13). The mesenchymally-derived tumors expressing high levels of TRE17 included osteoblastoma, myofibroma, and Ewing sarcoma (14). In the case of Ewing sarcoma, one out of the three primary tumor samples examined expressed high levels of TRE17. These observations raise the interesting possibility that TRE17 may function as a mesenchymal-selective oncogene *in vivo*. That said, TRE17's often cited involvement in Ewing's sarcoma pathogenesis is not yet well supported by any published studies. More extensive analysis of patient samples is required to determine whether TRE17 overexpression is a recurrent feature of Ewing sarcoma or any of these other tumors, and whether/how it contributes to the malignant phenotype (using knockdown approaches, for example). Nevertheless, one neoplasm in which TRE17 plays a clear etiological role is ABC, as evidenced by its recurrent translocation.

3.2. TRE17 and Aneurysmal Bone Cyst

ABC is a pediatric bone tumor that represents approximately 1-5% of all primary bone tumors (16, 17). Patients are typically diagnosed in the first two decades of life. Though considered benign, ABCs can nevertheless recur locally and cause extensive bone destruction. This can result in pain, swelling, deformity, neurological symptoms, and pathologic fracture. While the vast majority of cases occur in bone, ABCs can also arise in soft tissues, where they have an identical histology (18, 19). The majority of patients are treated by surgical curettage, but surgical excision or resection may be performed for recurrent tumors. In some instances ABCs can be difficult to excise and cause important morbidity for patients.

Though first described over 60 years ago, the etiology of ABC has remained obscure. The most widely accepted model has been that ABCs are reactive lesions, arising from a local circulatory disturbance leading to markedly increased venous pressure, and development of a dilated and enlarged vascular bed within the affected bone area (16, 17). This is proposed to cause hemorrhage and osteolysis, and further expansion of the lesion. Consistent with such a reactive origin, approximately 20-30% of ABCs occur subsequent to trauma or a pre-existing bone tumor, and are thus termed secondary ABC. However, the majority of ABCs are primary, arising *de novo* with no precursor lesion or history of trauma. A reactive origin for primary ABC is countered by the recent discovery that translocation of *TRE17* occurs in almost 70% of cases, pointing instead to a neoplastic origin (10, 11, 14, 18, 20, 21). Five different fusion partners of *TRE17* have been identified: cadherin 11 (*CDH11*), *THRAP150*, *ZNF9*, Osteomodulin (*OMD*), and collagen 1A1 (*COL1A1*) (14).

In every case, the translocation resulted in promoter swapping, where the highly active promoter of the translocation partner was fused to the first coding exon of TRE17, resulting in its transcriptional upregulation (10, 11, 14). Notably, *TRE17* is absent or expressed at very low levels in most normal adult human tissues except for testes (13).

These observations strongly suggest that *TRE17* overexpression plays a key role in the etiology of ABC. ABCs are complex lesions consisting of multiple blood-filled cysts, separated by fibrous stromal areas containing spindle cells, inflammatory cells, and numerous capillaries. Strikingly, *TRE17* translocation occurs exclusively in spindle cells within the fibrous stroma. Thus, although *TRE17* rearrangement is likely an early pathogenic event, there almost certainly must be an ensuing secondary reactive component. The precise identity of the cell affected by translocation is not known, and this remains one of the most important issues in dissecting ABC pathogenesis. Identifying the cell of origin is a common hurdle in the study of tumors harboring cytogenetic abnormalities. This is well exemplified by Ewing sarcoma, a malignant bone tumor that expresses both mesenchymal and neuroectodermal markers, and whose origin has only recently has been linked to mesenchymal stem cells (22, 23). Lineage analysis of the *TRE17*-rearranged cells in ABC has been extremely challenging, due in large part to the complexity of these tumors. As detailed above, they are cellularly heterogeneous and the cells harboring TRE17 rearrangement make up a variable percentage of the total tumor population. Furthermore, when primary tumors are cultured *in vitro*, the *TRE17*-rearranged cells are rapidly outgrown by contaminating fibroblasts. In addition, the cells harboring *TRE17* translocation/overexpression are morphologically indistinguishable from the non-expressing fibroblasts, precluding their isolation by methods such as laser capture microdissection (unpublished observations, A.M.O. and M.M.C.).

These obstacles have necessitated the development of ectopic expression systems that optimally mimic the cellular context in ABC. Although the cell of origin has not been definitively identified, an important clue is provided by the identity of *TRE17*'s fusion partners, whose promoters drive *TRE17* expression. All of *TRE17*'s partners are highly active in mesenchymal lineages such as fibroblasts and osteoblasts (the cell lineage responsible for bone formation) (10, 11, 14). Indeed, one fusion partner, *OMD*, is expressed exclusively in osteoblasts. Furthermore, osteoid (the organic component of bone matrix produced by osteoblasts) is commonly present in these lesions. Together, these observations suggest that the *TRE17* translocations occur in a mesenchymal precursor in the process of differentiation toward the fibroblast or osteoblast lineage. Recent studies demonstrate that multiple molecular and histological features of ABC are indeed recapitulated when TRE17 is ectopically expressed in immature osteoblasts (24), validating this as a promising model system for dissecting TRE17's mechanism of action in ABC pathogenesis.

3.3. Physiological Function of TRE17

While little is known about the pathological functions of TRE17 in ABC, even less is known about its role in normal physiology. This paucity of information derives from the fact that expression of TRE17 is highly restricted, and tractable systems for studying its function have been difficult to establish. First, among normal adult human tissues, TRE17 is absent or expressed at very low levels in most tissues except testes (13). Publicly accessible databases (BioGPS) suggest that within testes, TRE17 is expressed in a variety of cell types (including Leydig, interstitial, and germ cells). However, this expression has not been independently confirmed. TRE17 is also highly expressed in human fetal brain, rendering isolation/analysis of tissue unfeasible. Second, TRE17 is a primate-specific gene (13), precluding generation of a knockout mouse to determine its function during development *in vivo*. Together, these factors have conspired to hamper dissection of TRE17's functions in normal physiology.

Despite these challenges, two studies have provided hints at TRE17's function in humans, in particular during neuronal development, consistent with its high expression in fetal brain. In the first study, a patients with the autism spectrum disorder Asperger's syndrome was found to have the balanced translocation t(13;17)(q14;p13) (25). The breakpoint was mapped to a 25-30 kb region within the *TRE17* gene. The translocation was not mapped more precisely nor was the fusion partner identified, so it is not known whether the rearrangement led to generation of a novel chimeric protein, loss of TRE17 expression, or increased expression. Attempts at examining TRE17 expression levels in this patient were not informative due to technical limitations (25). Translocations of 17p13 have not previously been reported in Asperger's syndrome; however, it would be of great interest to determine whether TRE17 expression is altered in patients with this disorder, independent of cytogenetic abnormalities involving its locus.

In a second report, a child with mental retardation was identified with the constitutional translocation (15;17)(p12;p13.2), with the breakpoint mapping to a ~40 kB region within the *TRE17* locus (26). Additional fluorescence *in situ* hybridization (FISH) revealed additional signal of the telomeric region of 17p in the distal short arm of chromosome 15, resulting in duplication of 17pter. The child exhibited global developmental delays, and subtle facial dysmorphic features. Computed tomography (CT) and magnetic resonance imaging (MRI) revealed a number of brain abnormalities, including mild ventricular enlargement secondary to reduced periventricular white matter in the parietal lobes. In addition, the anterior commissure was significantly reduced in size. In this case again, the consequence of translocation (i.e. increased or loss of TRE17 expression, or generation of a chimeric protein) was not determined. Nevertheless, the identification of two patients with translocation breakpoints mapping to *TRE17* (one with Asperger's syndrome, and the other with mental

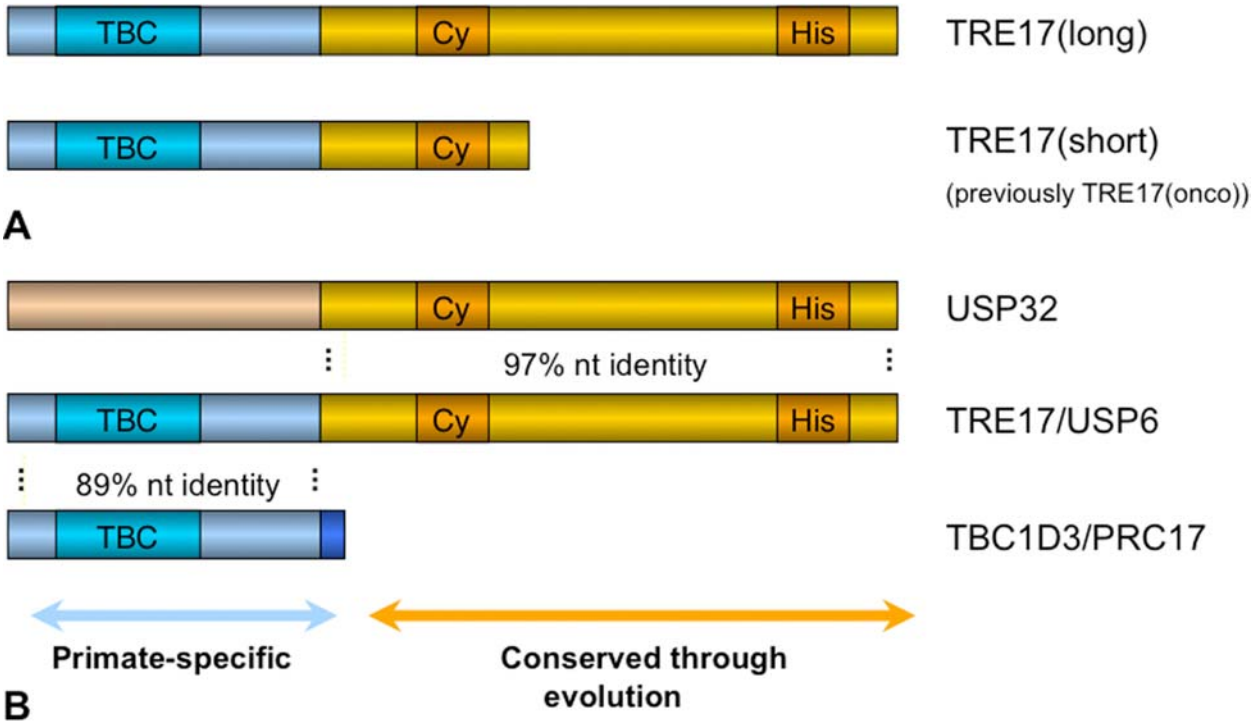

Figure 1. A) Domain structure of TRE17 isoforms. TBC, TBC domain; C and H, cysteine and histidine subdomains of the USP domain. Note: TRE17(short) was previously termed TRE17(onco) (5, 27, 29). B) TRE17 and its ancestor genes. TRE17 arose from the segmental duplication of two ancestor genes, *USP32* and *TBC1D3*, which are evolutionarily conserved and primate-specific, respectively. Percent nucleotide (nt) identity between TRE17 and its ancestors is indicated.

retardation) suggests that dysregulation of TRE17 contributes to neurologic developmental abnormalities.

Interestingly, analysis of the genomic architecture of human chromosome 17 has revealed a number of striking features, including key differences with its mouse ortholog (the distal half of chromosome 11) (27). First, human chromosome 17 has undergone extensive intrachromosomal rearrangements, whereas the mouse chromosome has been extremely stable. Second, the human chromosome has a high density of segmental duplications, while the mouse chromosome has a very low density. These segmental duplications correspond closely to sites of structural rearrangement, highly suggestive of a link between duplication and rearrangement. Notably, the region of chromosome 17 that comprises *TRE17* is one of these rearrangement-susceptible sites (26, 27). Thus, the *TRE17* locus represents a region of genomic instability that contributes to tumorigenesis and developmental abnormalities.

3.4. Structure and evolution of *TRE17* and its ancestor genes

TRE17 encodes two functional domains, an amino-terminal TBC domain, and a carboxy-terminal USP domain consisting of so-called cysteine and histidine subdomains, both of which are essential for catalytic activity (Figure 1A). Two splice variants differing in the presence of an exon have been described, giving rise to two polypeptides termed TRE17(long) and TRE17(short) (previously termed TRE17(onco)) (28). These isoforms are identical through their first 773 amino acids, but TRE17(short) is truncated shortly thereafter and is thus catalytically inactive as a USP. In contrast, TRE17(long) encodes a functional de-ubiquitinating enzyme (29, 30).

As mentioned above, *TRE17* is a hominoid/primate-specific gene. It is estimated to have emerged 21-33 million years ago, through the chimeric fusion of two ancestor genes, *USP32* and *TBC1D3* (13) (Figure 1B). *USP32* is evolutionarily conserved, while *TBC1D3* is generated through the primate-specific segmental duplications described above. Study of TRE17's ancestral genes has been limited, but both have been implicated in tumorigenesis:

USP32: TRE17 and USP32 possess 97% nucleotide identity within their USP domains, but contain divergent amino-termini. USP32 encodes potential signals for myristoylation and isoprenylation at its N- and C-termini, respectively, although the functionality of these motifs is not known. Recent work reveals that USP32 decorates the Golgi apparatus (15), contrasting it to TRE17, which localizes to the cytoplasm, endosomes, and plasma membrane (7, 28). Thus, despite the high degree of conservation between their USP domains, they likely target distinct substrates.

USP32 is located on chromosome 17q23, and is part of a breast-cancer associated amplicon that is amplified in 20% of HER2-positive breast tumors (31). *USP32* was

also identified as a member of a cohort of 81 genes whose copy number predicts metastatic capability of estrogen receptor-positive breast cancers (32). A very recent study confirmed elevated expression of *USP32* in 22% of primary breast tumors, and 50% of breast cancer cell lines (15). Furthermore, siRNA-mediated depletion of *USP32* from the MCF7 breast cancer line caused a reduction in their proliferation and migration rates. The only other functional study of USP32 has been on its presumed *Caenorhabditis elegans* ortholog, Cyk3, which is proposed to play a role in cytokinesis (33). Substrates for USP32 and Cyk3 have yet to be identified.

TBC1D3: TRE17 and TBC1D3 are highly homologous, sharing 81% amino acid identity over their N-terminal 499 residues. The *TBC1D3* gene resides on chromosome 17q12, a genomic region containing eight highly related paralogs that potentially encode six variant isoforms whose expression varies among normal human tissues and cancer cell lines (34).

TBC1D3 was originally identified based on its role in prostate cancer, hence its alternative name of prostate cancer gene 17 (PRC17) (35). The *TBC1D3* gene is amplified in 15% of prostate cancers, and highly expressed in approximately half of metastatic prostate tumors. Elevated expression has also been detected in primary breast tumors and breast cancer cell lines (35). Furthermore, overexpression of TBC1D3 is sufficient to induce transformation of NIH3T3 fibroblasts, as measured by mitogen-independent growth, focus formation, and tumor formation in nude mice (35). Recent work reveals that TBC1D3 overexpression causes dysregulation of epidermal growth factor receptor (EGFR) trafficking and signaling (36), providing a possible mechanism contributing to transformation.

Thus, both *TRE17* and its ancestor genes have been implicated in human neoplasia, albeit in different tumor types. While *USP32* and *TBC1D3* are amplified and overexpressed in epithelial tumors (breast and/or prostate), *TRE17* is translocated and/or overexpressed in mesenchymal tumors. These observations suggest that despite the extensive homology of *USP32* and *TBC1D3* with *TRE17*, these proto-oncogenes possess discrete functions in human tumorigenesis, perhaps acting in a cell-type selective manner. Indeed, functional studies thus far confirm that TBC1D3 and USP32 have cellular/molecular activities distinct from the TBC and USP portions of TRE17, respectively (see Section 5.1 for further details).

4. TARGETS/BINDING PARTNERS OF TRE17

In this section, the binding partners of TRE17 will be introduced, and a brief description of their molecular functions will be provided. Section 5 will speculate on how these binding partners might contribute to the cellular processes regulated by TRE17. A summary of Sections 4 and 5 is presented in Figure 2.

4.1 TBC Domain Targets

TBC domains generally function as GAPs for Rab GTPases. However, TRE17 is devoid of GAP activity, as it lacks two highly conserved residues that are essential for catalysis in other TBC domains (7, 8, 37). In addition, the region C-terminal to the TBC domain appears to lack secondary structural elements required for GAP function (8). Despite this lack of catalytic activity, the TBC domain of TRE17 retains the ability to interact with small GTPases. Surprisingly, however, it binds to Arf6, a member of a distinct subfamily of GTPases, and promotes its activation *in vivo* (7). In addition, a recent genome-wide yeast two-hybrid screen identified Rab33B, a resident Golgi protein, as a potential binding partner for the TBC domain of TRE17 (38-40). The physiological relevance and consequences of this interaction remain to be determined.

Arf6 has diverse cellular functions, including regulation of phospholipid metabolism, endocytic trafficking, actin remodeling, and mitogenic signaling, which mediate its role in promoting cell motility and invasiveness (41-50). Studies have begun to explore TRE17's role in modulating these Arf6-regulated processes, and their potential contribution to ABC pathogenesis (see Section 5.1).

4.2. USP Substrates

Two key questions regarding TRE17's mechanism of action in ABC are: what is the role of its USP domain, and what are its substrates? Analyzing expression of the isoforms in primary ABC tumors has not been particularly informative, since both TRE17(long) and TRE17(short) were found to be expressed (as determined by RT-PCR of a small sample set, using primers that distinguish between the two isoforms; unpublished observations, A.M.O. and M.M.C.). More direct evidence was recently provided by a xenograft study, which demonstrated that USP activity is essential for tumor formation ((24) and discussed in detail in Section 6.1).

These results confirm that identification of TRE17 USP substrate(s) is essential. To date, no direct substrates have been determined. However, recent work has shown that TRE17 is mono-ubiquitinated and promotes its own de-ubiquitination *in vivo* (30). This is suggested by the observation that catalytically inactive alleles of TRE17 alleles (TRE17(short) as well as a point mutant of the catalytic cysteine residue, TRE17(long)/USP-) exhibit greatly elevated steady state levels of mono-ubiquitination compared to TRE17(long) *in vivo*. However, direct auto-de-ubiquitination using purified TRE17 was not demonstrated *in vitro*. Therefore, whether TRE17 catalyzes its auto-de-ubiquitination or functions through an intermediary DUB remains to be determined.

The function of TRE17 mono-ubiquitination also remains unclear. Mono-ubiquitination of proteins can regulate their endocytic trafficking. Consistent with this, alleles of TRE17 that lack USP activity (and thus have relatively high levels of mono-ubiquitination) exhibit decreased plasma membrane association and enhanced localization to intracellular endosomal compartments in epithelial cells (7). Mono-ubiquitination can also affect protein stability by promoting lysosomal targeting. However, the stability/steady state levels of the various

TRE17 alleles was not found to correlate with their degree of mono-ubiquitination (7, 30).

Thus, identifying heterologous targets of TRE17's USP domain remains a crucial goal. The recent identification of effector pathways activated by TRE17 invokes potential candidate substrates to be explored (Section 5.3).

4.3. Additional binding partners

4.3.1. Calmodulin

The ubiquitous calcium-binding protein calmodulin (CaM) is a binding partner for TRE17 (30). Calmodulin (CaM) binds directly to TRE17 in a Ca^{2+}-dependent manner, and co-immunoprecipitation of TRE17 with endogenous CaM has been demonstrated *in vivo*. The CaM interaction site maps to the C-terminus of the TBC domain. Association of TRE17 with CaM is not required for binding of Arf6 to the TBC domain. Interestingly, however, point mutants that ablate CaM binding exhibit greatly reduced mono-ubiquitination, suggesting that CaM might either promote TRE17's association with the ubiquitin ligase that catalyzes this modification, or inhibit TRE17's USP activity. Once TRE17 substrates are identified, this latter possibility may be explored.

4.3.2. Myl2 and ANKRD44

Myl2 and ANKRD44 were identified as TRE17 binding partneres in a yeast two-hybrid screen using the TBC domain of TRE17 as bait (51). Myl2 (myosin regulatory light chain 2; a.k.a. MLC2) functions as the regulatory subunit of myosin, and is highly homologous to CaM (52). ANKRD44 is an ankyrin repeat domain protein that functions as the regulatory subunit B of protein phosphatase 6 (PP6) (53). Recombinant GST fusions of Myl2 and ANKRD44 were able to pull down *in vitro* translated TRE17 TBC domain (51). Furthermore, epitope-tagged forms were shown to co-immunoprecipitate with the isolated TBC domain when overexpressed in HEK293 cells.

While binding of these proteins to full length TRE17 has not yet been confirmed, one may speculate on their possible roles in TRE17's functions. It is reasonable to propose that Myl2 binds to TRE17 at the same site as CaM, given their high degree of homology (52). Myl2 functions as the regulatory subunit of myosin, a key regulator of actin-based contraction in non-muscle cells. Thus, Myl2 could conceivably contribute to the actin remodeling functions of TRE17 (see Section 5.2). ANKRD44 is a putative regulatory subunit for PP6 (53), which has been implicated in regulation of the DNA damage response (54). Notably, PP6 has also been shown to regulate NFκB (55, 56), a recently identified target of TRE17, and might thus function as a mediator in activating this transcription factor (see Section 5.3 for further details).

5. CELLULAR PATHWAYS AND RESPONSES ACTIVATED BY TRE17

TRE17 has been shown to initiate several cellular responses, as summarized in Figure 2 and detailed in this section. How these pathways and responses might contribute to ABC pathogenesis is further considered in Section 6.

5.1. Arf6 and Endocytic trafficking

Arf6 regulates diverse cellular functions, including endocytic trafficking, actin remodeling, and mitogenic signaling (41-46). Amongst these functions, TRE17's role in endocytic trafficking has been investigated in mechanistic detail. The Arf6 endocytic pathway represents a pathway that is distinct from the more widely studied clathrin-dependent route in many cell types (42, 43, 57). Trafficking between the plasma membrane and endosomes of this pathway is controlled by the GTPase cycle of Arf6, and its ability to regulate phospholipid metabolism. The Arf6 pathway controls the trafficking of important regulatory molecules, such as β1 integrin and E-cadherin.

TRE17 regulates trafficking through the Arf6 pathway (7). TRE17 binds directly to Arf6 specifically in its GDP-bound form, and overexpression of TRE17 induces activation of Arf6 *in vivo*. Conversely, siRNA-mediated depletion of TRE17 reduces basal levels of active Arf6. TRE17's ability to promote Arf6 activation does not require USP activity, as an N-terminal construct lacking the entire USP domain is sufficient for this response. However, binding of Arf6 to TRE17 is required, since a triple point mutant of the TBC domain (TRE17(A6-)) that ablates interaction fails to activate Arf6 *in vivo* (58). The mechanism by which TRE17 elicits Arf6 activation has not been elucidated, but likely involves regulation of Arf6's subcellular localization and/or access to GEFs. TRE17 also causes phenotypic alterations that mimic hyper-activation of Arf6, including Arf6 endosome enlargement and perturbations in β1 integrin/MHCI trafficking (7).

Interestingly, recent work has shown that TRE17 is required for West Nile Virus (WNV) infection (59). TRE17 was identified in a genome-wide siRNA screen aimed at identifying cellular factors required for WNV internalization. These studies showed that depletion of TRE17 prevented entry of WNV into HeLa cells. While this work did not identify the molecular basis for TRE17's involvement, a role in virus internalization is consistent with its role in regulating endocytic trafficking.

Of note, although TRE17 and TBC1D3 are highly related within their TBC domains, TBC1D3 does not bind to Arf6 or induce its activation *in vivo* (60). Several reports demonstrate that it nevertheless regulates endocytic trafficking. Frittoli *et al.* reported that overexpression of TBC1D3 is sufficient to stimulate macropinocytosis, and that its knockdown by siRNA attenuates macropinocytosis induced by EGF (60). Wainszelbaum and colleagues showed that TBC1D3 modulates trafficking of the EGFR: overexpression of TBC1D3 delays ligand-induced downregulation of EGFR, resulting in enhanced activation of Ras, Erk, and Akt, while its knockdown has the opposite consequences (36). TRE17 does not modulate EGFR trafficking (unpublished observations, M.M.C.). The mechanism by which TBC1D3 functions, and whether this involves binding of a

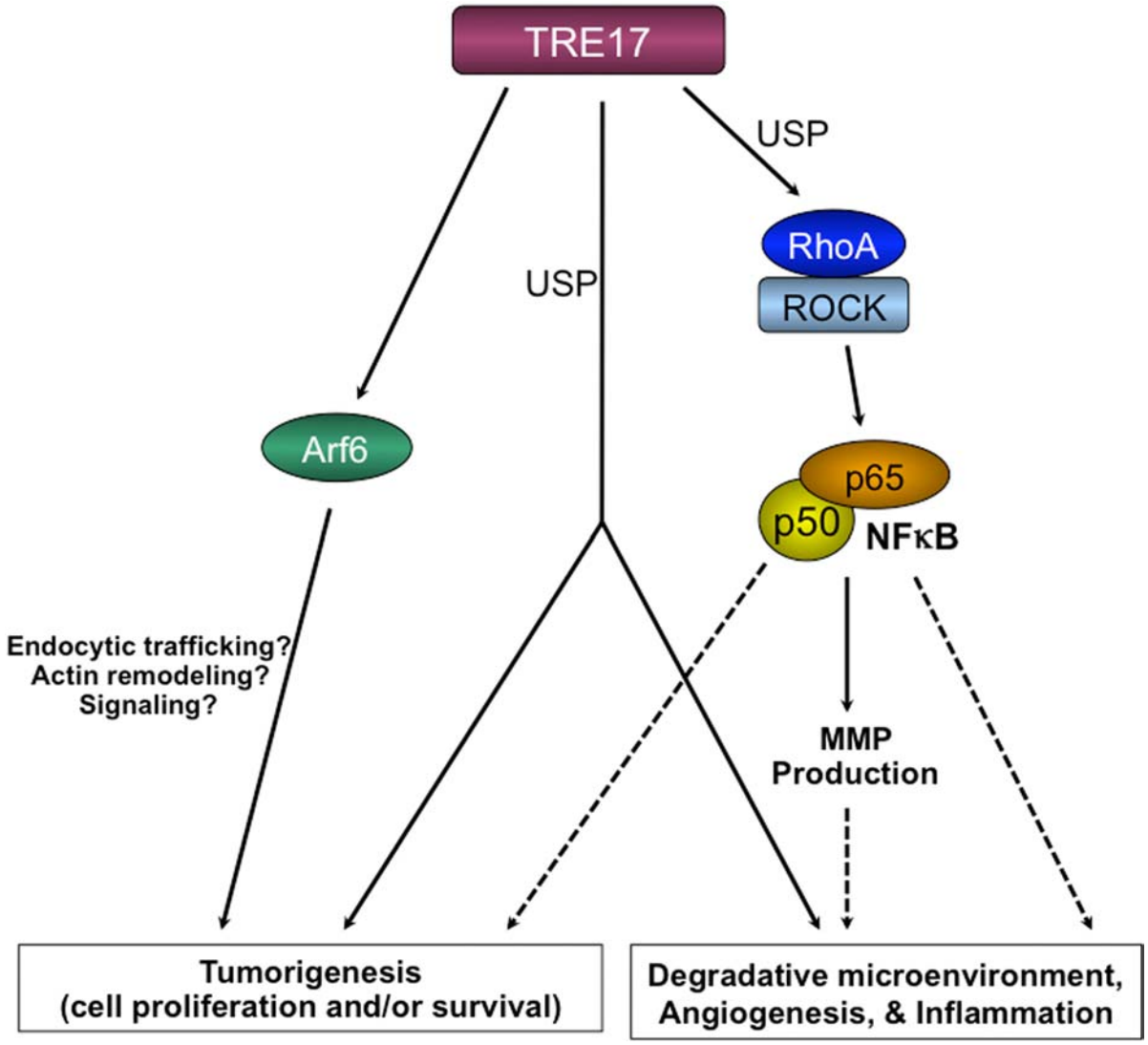

Figure 2. Summary of signaling pathways activated by TRE17, and proposed mechanism of ABC pathogenesis. See text for details. Solid arrows indicate processes that have been demonstrated; dashed arrows indicate proposed functions that remain to be tested. Aside from inducing RhoA/ROCK-dependent activation of NFκB/MMP induction, TRE17's USP domain may promote tumor cell survival, angiogenesis, and inflammation through additional mechanisms. The mechanism by which Arf6 promotes tumorigenesis is unknown, but could potentially involved its regulation of endocytic trafficking, actin cytoskeletal remodeling, and/or signaling.

GTPase to its TBC domain, are not known. While initial studies suggested that it might function as a weak GAP for Rab5 *in vitro* (35) it is unclear if it does so *in vivo,* since TBC1D3 lacks key catalytic residues.

5.2. Actin remodeling

As mentioned above, TRE17 cycles between the cytosol, plasma membrane, and endosomes. Growth factors such as EGF stimulate TRE17's recruitment to the plasma membrane, mediated by the Rho family GTPases, Cdc42 and Rac1 (28). Once recruited to the plasma membrane TRE17 induces reorganization of the actin cytoskeleton, specifically the accumulation of contractile actin filaments at the cell cortex (28). Overexpression of a TRE17 construct encoding the amino-terminal 447 amino acids (which contains the TBC domain) is sufficient to induce this response, indicating that its USP activity is not essential.

The mechanism by which TRE17 induces actin remodeling remains to be determined, but could involve several candidate effectors. One potential mediator is Arf6, which has been shown to regulate actin dynamics, in part through its effects on phospholipid metabolism and crosstalk with Rho family GTPases (42, 43, 45). Consistent with this possibility, the minimal construct capable of inducing actin remodeling was also sufficient to activate Arf6 *in vivo* (7). The putative TRE17-binding

protein Myl2, through its regulation of myosin, could also contribute to the cytoskeletal alterations induced by TRE17 (52). In fact, the cortical actin filaments induced by the N-terminal TRE17 construct are most consistent with an effect actomyosin-based contraction. A third possible candidate mediator is RhoA, which was recently shown to be activated by TRE17 (24). RhoA, through its effector kinase ROCK, also stimulates actin contractility and formation of stress fibers through regulation of MLC/myosin (61-65).

5.3. NFκB activation and Matrix Protease Production

The work describing TRE17's effects on endocytic trafficking and actin remodeling pre-dated the discovery that the cells affected by *TRE17* translocation in ABC are mesenchymal. Thus, those studies were performed in HeLa cervical carcinoma cells, which endogenously express low levels of TRE17. More recent work has begun to examine the effects of TRE17 in cells that might be more relevant to understanding its pathogenic functions in ABC, namely pre-osteoblasts. This study examined the effects of TRE17 overexpression in two pre-osteoblastic cell lines, human hFOB1.19 and murine MC3T3 cells (24).

ABCs are characterized by osteolysis, inflammatory recruitment, and extensive vascularization, processes in which matrix proteases play a prominent role. Indeed, previous immunohistochemistry (IHC) showed that the fibrous stromal component of ABC (where the *TRE17*-rearranged cells reside) stains strongly for MMP-9. This prompted investigators to explore whether TRE17 regulates the production of matrix metalloproteinases (MMPs) (24). Using an unbiased antibody-based approach to screen a panel of MMPs, TRE17(long) was found to induce the production of MMP-9 and MMP-10 when expressed in hFOB1.19 or MC3T3 cells, in the absence of any serum-derived factors. USP activity was required, since TRE17(short) and TRE17(long)/USP- failed to induce MMP-9/-10 production. However, Arf6 binding/activation was dispensable, since the TRE17(A6-) mutant induced MMP production comparably to wild type TRE17(long). Further analysis revealed that TRE17 functioned by inducing transcription of MMP-9, through a mechanism involving activation of NFκB, a transcription factor with a central role in inflammation and transformation. In turn, activation of NFκB was mediated in part through activation of RhoA and its effector kinase ROCK (30) (Figure 2). Though previous IHC had documented the expression of MMP-9 in ABC, the cells responsible for its production had not been identified (66). Furthermore, it was unclear whether MMP-9 had a causal role in ABC pathogenesis, or was produced secondarily as a result of tissue damage and inflammation. The finding that TRE17 directly induces its production favors the former scenario.

It is worth pointing out that TRE17 was competent to activate NFκB/MMP-9 in mouse osteoblasts. Thus, despite the fact that TRE17 is not normally expressed in mice, it retains the ability to engage pathways that are evolutionarily conserved in murine cells. TRE17 also induced this entire pathway in HeLa cells (which are epithelial), indicating that this response is not dependent on the mesenchymal context. Therefore, unraveling the molecular basis for the selection for high TRE17 expression in mesenchymal tumors requires further investigation.

6. MECHANISMS OF TUMORIGENESIS

6.1. Tumorigenesis requires both TRE17's USP and TBC domains

TRE17's tumorigenic potential was also examined in the osteoblast-based studies above, xenografting the MC3T3 lines expressing TRE17 alleles into nude mice (24). The authors introduced the xenografts subcutaneously, rationalizing that although ABCs most commonly arise in bone, they can also occur in soft tissue, where they have an identical histology and clinical behavior (18, 19). TRE17(long)/MC3T3 induced the formation of tumors that reproduced multiple aspects of ABC: lesions were composed of a spindle cell population associated with a loose fibromyxoid matrix (24), highly reminiscent of ABC (10, 67). Tumors demonstrated extensive vascularization as well as blood extravasation, both of which are prominent features of ABC. Immunohistochemical analysis revealed an accumulation of MMP-9 around the TRE17-expressing cells, providing *in vivo* confirmation of the *in vitro* results, and reproducing results from patient samples (66).

Strikingly, MC3T3 cells expressing TRE17(short) or TRE17(long)/USP- failed to form tumors, demonstrating that TRE17's USP activity is essential for MC3T3 cell proliferation and/or survival *in vivo*. These results were unexpected, since no significant differences in cell proliferation or survival were observed between control and any of the TRE17-expressing MC3T3 cells *in vitro* (58). Even more surprisingly, TRE17(A6-) also failed to induce tumor formation (unpublished observations, M.M.C.), despite the fact that this mutant functioned comparably to WT TRE17(long) in all *in vitro* assays (24). Thus, the tumorigenesis assays revealed an additional function for both the USP domain and the TBC domain/Arf6 in MC3T3 cell proliferation and/or survival *in vivo*.

Notably, the tumorigenesis studies using MC3T3 cells directly contradict the original characterization of TRE17 using NIH3T3 fibroblasts, which showed that TRE17(short) but not TRE17(long) induced tumor formation in nude mice (4) (hence prior designation of TRE17(short) as TRE17(onco)) (7, 28, 30). The reason for this disparity is still unclear, but one possibility is the use of different cell types. Such diametrically opposed results would be somewhat surprising, however, given that these two mesenchymal lineages are extremely similar (68). Furthermore, the promoters of TRE17 fusion partners (except for *OMD*) are highly active in both lineages, and thus either one could potentially represent the affected cell in ABC. One caveat to the NIH3T3-based study is that expression of the different isoforms was not confirmed in the cell lines used for the xenografts. Furthermore, histological/immunohistochemical analysis was not

performed to determine whether the tumors reproduced features of ABC. Another notable difference was the latency of tumor formation: tumors appeared after 1-2 weeks with the MC3T3 xenografts, but required at least 4 weeks with the NIH3T3. Thus, while it remains possible that use of different cell types accounts for the varied tumorigenic potential of TRE17(long) vs. TRE17(short), additional studies in NIH3T3 are necessary. However, since the MC3T3 studies indicate that the truncated isoform is not oncogenic, this peptide was re-designated TRE17(short).

6.2. Cellular functions induced by TRE17: relevance to ABC and neoplasia

Among the cellular functions attributed to TRE17 in Section 5 above, which ones are most relevant to TRE17's tumorigenic potential and ABC pathogenesis? Much work remains to be done to address this issue. However, it seems likely that NFκB activation/MMP production is an important effector pathway. As mentioned, prior work has confirmed that ABCs stain strongly for MMP-9, making it a likely physiological target of TRE17 *in vivo* (66). There are multiple means by which MMP-9, as well as MMP-10, could contribute to ABC pathogenesis. Both of these MMPs have previously been implicated in remodeling/degradation of bone matrix, under physiological and pathological conditions (66, 69-77). MMP-9 and MMP-10 have multiple substrates including other MMPs, which induces their zymogenic activation (70, 78, 79), potentially further contributing to the degradative microenvironment in ABC. They can also proteolyze components of the extracellular matrix, which not only degrades the ECM but also liberates matrix-bound growth factors that can promote inflammation and angiogenesis (70). For example, numerous studies indicate that MMP-9 plays an important role in the release of vascular endothelial growth factor (VEGF) from bone and cartilage matrix, thereby promoting angiogenesis and vascular permeability (80-83). MMP-9 is believed to further contribute to angiogenesis by degrading the endothelial and interstitial matrix to facilitate endothelial cell migration and branching. Thus, the functions of MMP-9 (especially its role in angiogenesis) are fully consistent with the histological features of ABC, which is enriched in vascular elements and extravasated blood.

In addition to mediating induction of MMP-9, NFκB may have additional roles in ABC pathogenesis. NFκB plays a central role in the inflammatory response, in part by inducing the expression of inflammatory cytokines (84). Thus, it is tempting to speculate that activation of NFκB by TRE17 functions not only to promote establishment of a degradative microenvironment (through induction of matrix proteases), but also an inflammatory one (through the induction of cytokines). In fact, histological analysis of ABCs has shown that *TRE17*-rearranged cells are often adjacent to clusters of multi-nucleate giant cells (10), which develop from hematopoietic precursors in response to cytokines (85-89). Furthermore, the regions surrounding the *TRE17*-rearranged cells exhibit a high proliferative index (10, 18). Thus, it is speculated that TRE17 likely induces the production of a variety of factors to drive the inflammation, angiogenesis, and proliferation that characterize these lesions, with a pivotal role for NFκB in this process. Indeed, it is further posited that TRE17's paracrine functions play an essential role in ABC pathogenesis.

It remains to be determined how TRE17's effects on Arf6, RhoA, actin remodeling and endocytic trafficking contribute to its tumorigenic potential. Although activation of Arf6 and RhoA are often associated with enhanced cell migration and invasiveness, TRE17(long) did not affect these properties in the MC3T3 cells when assayed *in vitro* (58). Though somewhat surprising, this is nevertheless consistent with the clinical observation that ABCs are not metastatic, with the exception of one recently described case (90). Also unexpected was the finding that TRE17 regulates MMPs on a transcriptional level (24). Recent work has shown that Arf6 induces matrix proteases, but that it functions post-translationally to promote their exocytosis and/or release from cells (44, 47-49). Thus, it was anticipated that TRE17 might function through Arf6 to regulate MMP trafficking, particularly since previous work had suggested a role for TRE17 in endocytic recycling (7). However, the osteoblast studies clearly demonstrate that TRE17 functions predominantly through transcription, and does not require its ability to activate Arf6. Thus, the pathways and responses activated downstream of Arf6 may vary depending on the cellular context. Even though Arf6 is dispensable for TRE17's induction of NFκB and MMP-9 *in vitro*, it is essential for tumor development *in vivo*, (unpublished observations, M.M.C.), and determining the underlying mechanism remains an important issue. It is conceivable that TRE17's activation of Arf6 and RhoA modulates adhesion/integrin-based signaling, which is known to impact cell survival and proliferation (91, 92).

7. CONCLUDING REMARKS

7.1. Novel therapeutic strategies

The recent insights into TRE17's molecular functions and mechanism of tumorigenesis have opened up potential new avenues for treatment of ABC. Currently, the mainstay of ABC treatment is surgical curettage (16). The studies of Ye *et al.* raise the possibility that selective inhibitors of ROCK and NFκB might be used in combination for management of ABC (24). Notably, inhibitors for these targets are already currently in use or are being avidly pursued for the treatment of malignancy, inflammation, and other diseases (93-95). Their work also indicates that USP-specific inhibitors of TRE17 would serve as effective therapeutic agents, since USP activity was required not only for NF□B/MMP induction, but also for tumor cell survival in vivo. Furthermore, given TRE17's highly restricted range of expression, a USP-specific inhibitor would represent an ideal chemotherapeutic agent with minimal toxicity in normal tissues. One caveat, however, would be that such an inhibitor might also act on USP32. Thus, alternative approaches, such as development of agents that inhibit TRE17's binding to Arf6 or Arf6 activation, should also be considered.

7.2. Key issues for the future

A number of key issues remain for understanding TRE17's role in the pathogenesis of ABC. First, it is essential to confirm whether the pathways activated in the MC3T3 model reflect events observed in patients, particularly the activation of NFκB. While activation of NFκB is fully consistent with the inflammatory and degradative nature of ABC, whether it is a target of TRE17 *in vivo* requires validation in primary tumor samples. Given the extensive ongoing efforts to develop NFκB inhibitors, confirming its role in ABC pathogenesis might be a promising route to a novel therapy.

Another important issue relates to determining the identity of the cell affected by TRE17 translocation. While MC3T3 xenografts recapitulated a significant number of features of early stage ABC (16), they did not reproduce the full spectrum of characteristics of human tumors. For example, TRE17(long)/MC3T3 tumors remained as solid masses, and did not develop into the multi-cystic lesions typically seen in ABC (although 5-10% of cases are indeed solid) (96). One possible explanation is that although *TRE17* overexpression is sufficient to initiate tumor formation, additional events may be required to fully reproduce these complex lesions. However another possibility, which is not mutually exclusive, is that MC3T3 cells do not precisely mimic the cellular context of the *TRE17*-translocated cells in ABC. Other candidates for the cell type affected by *TRE17* rearrangement are fibroblasts and mesenchymal stem cells; future studies should explore these additional cell models for their ability to better recapitulate ABC pathogenesis. Ideally, this should be coupled with development of approaches to isolate the *TRE17*-rearranged cells from primary tumors to determine their lineage of origin.

An additional factor to consider in improving an animal model of ABC is the host background, in particular to improve reproduction of the inflammatory aspect. While some inflammatory cells were present in the MC3T3 tumor xenografts, the extent of their infiltration was less pronounced than typically seen in patients. A likely contributing factor is the severe deficiency of mature T cells in nude mice. T cells play an essential role in modulating the inflammatory response through the production of cytokines. Thus, use of immunocompetent mice (such as the C57BL/6 strain, from which the MC3T3 cell line was derived) should be explored to better reproduce the inflammatory component of ABC. Further refinement of this mouse model would be instrumental for identifying the TRE17 effector pathways critical for ABC pathogenesis, and for ultimately testing therapeutic agents.

On a mechanistic level, a key goal that remains is the identification of substrates of TRE17's USP domain. The work of Ye *et al.* demonstrated that TRE17's USP activity was essential for NFκB activation, MMP induction, and tumorigenesis. The identification of NFκB as a target of TRE17 was particularly informative, since numerous upstream regulatory components of this pathway are modified by ubiquitin, providing multiple potential candidate substrates to explore (97, 98).

On a final note, although ABC is a rare tumor, information gleaned from analysis of TRE17's functions in this context may be more broadly applicable to other mesenchymal neoplasms in which it is highly expressed, such as Ewing sarcoma. Furthermore, such studies may provide insights into novel and unique regulatory mechanisms governing cell proliferation, tumorigenesis, and inflammation that have emerged during human evolution.

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