Anthrax Toxin Receptor (ATR/TEM8) is Highly Expressed in Epithelial Cells Lining the Toxin’s Three Sites of Entry (Lung, Skin, and Intestine):

Implications for the Pathogenesis of Anthrax Infection

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Abbreviations: ATR, anthrax toxin receptor; TEM8, tumor endothelial marker; PA, protective antigen; LT, lethal toxin; ET, edema toxin; CMG2, capillary morphogenesis protein 2.
Abstract

Anthrax is a disease caused by infection with spores from the bacteria *Bacillus anthracis*. These spores enter the body, where they germinate into bacteria and secrete a tripartite toxin that causes local edema and, in systemic infections, death. Recent studies identified the cellular receptor for anthrax toxin (ATR), a type I membrane protein. ATR is one of the splice variants of the tumor endothelial marker 8 (TEM8) gene. ATR and TEM8 are identical throughout their extracellular and transmembrane sequence, and both proteins function as receptors for the toxin. ATR/TEM8 function and expression has been associated with development of the vascular system and with tumor angiogenesis. TEM8 is selectively upregulated in endothelial cells during blood vessel formation and tumorigenesis. However, selective expression of TEM8 in endothelial cells contradicts the presumably ubiquitous expression of the receptor. To resolve this controversial issue, we evaluated the distribution of ATR/TEM8 in a variety of tissues. For this purpose, we generated and characterized a novel anti-ATR/TEM8 polyclonal antibody. Here, we show that this novel antibody recognizes all three ATR/TEM8 isoforms, which are widely and differentially expressed in various tissue types. We found that ATR/TEM8 expression is not only associated with tumor endothelial cells, as previously described. Indeed, ATR/TEM8 is highly and selectively expressed in the epithelial cells lining those organs that constitute the anthrax toxin's sites of entry, i.e. the lung, the skin, and the intestine. In fact, we show that ATR/TEM8 is highly expressed in the respiratory epithelium of the bronchi of the lung, and is particularly abundant in the ciliated epithelial cells coating the bronchi. Furthermore, immunostaining of skin biopsies revealed that ATR/TEM8 is highly expressed in the epithelial keratinocytes of the epidermis. Finally, we show that the epithelial cells lining the small intestine strongly express ATR/TEM8 isoforms. This is the first demonstration that the ATR/TEM8 protein is highly expressed in epithelial cells, which represent the primary entry site for the anthrax toxin. These results suggest that the ATR/TEM8 expression pattern that we describe here is highly
relevant for understanding the pathogenesis of anthrax infection.
Anthrax is a disease caused by infection from spores of the bacteria *Bacillus anthracis* (16). Depending on the route of infection, the disease presents with three different clinical manifestations: cutaneous (in which infection occurs through the skin), gastrointestinal (in which infection occurs through ingestion of spores) and pulmonary anthrax (which occurs through inhalation of spores) (11). Pulmonary anthrax is the most severe form, which often leads to death.

In recent years, many important advances have been achieved in the anthrax field. These findings are expected to stimulate the development of new inhibitors against anthrax toxin (18, 23). The anthrax bacterium releases a toxin that is essential for lethal effects. This virulence factor composed of three proteins: protective antigen (PA, which binds to cellular receptors), lethal factor (LF, which is a protease), and edema factor (EF, which belongs to the adenyl cyclase class of proteins). The crystal structures of protective antigen, lethal factor, and edema factor have been solved (12, 20, 21). LF and EF are individually non-toxic, and require association with PA to enter the cytosol and to produce many of the symptoms of anthrax infection. The combination of PA and LF forms the lethal toxin, while the association of PA with EF forms the edema toxin. During cellular infection, PA binds anthrax toxin receptor and is cleaved at the cell surface by furin and/or a furin-like protease. Cleaved PA oligomerizes, and binds to EF or LF, or both. The complex is then internalized via endocytosis (1, 2, 14), trafficked to the acidic environment of endosomes, which promotes channel formation and translocation of LF or EF (10, 17).

The edema factor is an adenylate cyclase that induces a significant increase in the intracellular concentration of cAMP. Elevated levels of cAMP in the host cells alter water homeostasis, which leads to swelling and edema. The lethal factor is required for cell killing and morbidity. LF is a protease that cleaves members of the mitogen-activated
protein kinase kinase (MAPKK) family thereby disrupting three MAPK signaling pathways. The direct involvement of these pathways in LT killing remains to be shown (17).

The identification of cellular receptors for anthrax toxin represents valuable progress in understanding the molecular events involved in the intoxication mechanism, and in the development of anti-toxins. The receptor, termed ATR (anthrax toxin receptor), is a type I membrane protein, and is expressed at moderately high levels on the cell surface (6). The extracellular region of ATR contains a von Willebrand factor type A (VWA) domain, which may modulate protein-protein interactions. This VWA domain constitutes the direct binding site for PA. In addition, a MIDAS (Metal-Ion-Dependent-Adhesion-Site) motif, localized within the VWA domain, is important for toxin binding (5-7).

ATR is encoded by the tumor endothelial marker 8 (TEM8) gene. Three variants result from alternative splicing of the TEM8 gene. The long isoform (TEM8) is a transmembrane protein of 564 amino acids, with a long proline-rich cytoplasmic tail. The medium isoform (ATR) is a 368 amino acid protein, which possesses a short cytoplasmic tail, and diverges from the long isoform at the last four amino acids at the C-terminus. The long and medium isoforms are identical throughout the extracellular region, the putative transmembrane domain, and a portion of the cytoplasmic tail. As they both contain the VWA domain that binds to PA, they both function as PA receptors (6). On the contrary, the short isoform lacks any sequence for membrane attachment. As a consequence, this putative secreted protein does not function as a PA receptor (22). Interestingly, ATR/TEM8 is highly conserved in different species, and mouse and human homologues share 98% sequence identity in the extracellular domain (6).

Although the physiological function of ATR/TEM8 is still unknown, several lines of evidences suggest a role in the regulation of angiogenesis. In fact, several independent
investigators have shown that, in humans, TEM8 is preferentially expressed in endothelial cells within colonic tumors (9, 19, 24). In mice, TEM8 was shown to be highly expressed in tumor vessels, as well as in the vasculature, of developing embryos, but undetectable in normal tissues. Furthermore, a second protein was identified to function as an anthrax toxin receptor: the human capillary morphogenesis protein 2 (CMG2) (4) (15, 25). Currently, CMG2 is the protein most similar to ATR/TEM8. CMG2 and ATR possess common characteristics, including a type I transmembrane domain, and a VWA domain. These two proteins share 40% amino acid identity throughout their sequence, and 60% identity within their VWA domain. Interestingly, similarly to ATR/TEM8, CMG2 was shown to bind PA with its VWA domain. CMG2 is expressed in a variety of tissues, but it is up-regulated in human umbilical vein endothelial cells during the process of capillary formation. Taken together, these findings pinpoint that one key function of ATR/TEM8 may be the regulation of the neovasculature.

Prior to the molecular cloning of ATR, the PA binding receptor was tentatively localized to the basolateral surface of polarized epithelial cells. A series of elegant studies demonstrated that EF enters epithelial cells through the basolateral, but not the apical surface, suggesting that the receptor expression is restricted to the basolateral membrane of polarized epithelial cells. As such, the exact cellular distribution of ATR/TEM8 remains unknown (endothelial versus epithelial). For example, the expression of ATR/TEM8 in epithelial cells has not been reported to date.

In the current study, we have examined the tissue-specific and cellular distribution of ATR/TEM8 in a variety of normal mouse and human tissues. For this purpose, we generated and characterized a novel polyclonal antibody that selectively recognizes an extracellular epitope within the VWA domain of the ATR/TEM8 protein. The generation of this novel antibody allowed us to identify the cellular targets of anthrax toxin at the
molecular level.
Materials and Methods

**Materials.** Anti-Myc IgG (rabbit polyclonal) was obtained from Santa Cruz Biotechnology. Human lung and skin tissue micro-arrays sections were from Imgenex.

**Animal Studies.** For all these experiments, we used 3-month-old male wild-type mice in the C57Bl/6 background. Mice were housed and maintained in a barrier facility at the Albert Einstein College of Medicine, with 12-hour light/dark cycle and they had *ad libitum* access to chow (Picolab 20; PMI Nutrition International) and water. Mice were sacrificed, the lungs were removed and insufflated with 2 ml of 10% neutral-buffered formalin. Small intestine samples and skin biopsies were surgically removed and fixed in 10% neutral-buffered formalin for 24 hours, after which the samples were placed in 70% ethanol until processing. Tissue samples were paraffin-embedded, 4- to 5-µm sections were cut, and placed on Super-Frost Plus-slides (Fisher) for immuno-histochemistry (8).

**Construction of ATR/TEM8 cDNAs.** The cDNAs encoding the long, medium, and short isoforms of ATR/TEM8 were re-isolated and subcloned into TOPO, a mammalian expression vector (Invitrogen), according to manufacturer’s instructions. A Myc-tag epitope was placed at the C-terminus of each construct. For isolation of the cDNAs, we employed Human Quick Clone cDNAs (from Clonetech, Inc.) and the Platinum Taq High Fidelity polymerase (from Gibco, Inc.). Primers for amplification were designed based on the following three accession numbers: AF421380; NM032208; and BC012074. Also, a Kozak sequence was placed immediately ahead of the start site (GCC ACC ATG). The correctness of all clones was verified by DNA sequencing.

**Cell Culture and Transfection.** CHO cells were grown in RPMI supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% fetal calf serum. CHO cells were transiently transfected using the Lipofectamine transfection reagent (Invitrogen), as
per manufacturer’s instructions, and cellular expression was analyzed 36 h post-transfection.

**Antibody Production.** A polyclonal antibody to ATR/TEM8 was generated by immunization of New Zealand White rabbits with a synthetic peptide (residues 92-107; LMKLTEDREQIRQGLEC) corresponding to a sequence in the extracellular domain of human ATR/TEM8, containing an exogenously added C-terminal cysteine residue to facilitate maleimide conjugation to KLH. The resulting IgG's were purified from serum by ammonium sulfate precipitation, followed by resuspension in PBS and subjected to peptide affinity chromatography.

**Western Blotting.** Cells were lysed in hot sample buffer (13). Samples were then collected, and homogenized using a 26 g needle and 1 ml syringe. Murine tissue lysates were mixed with an appropriate volume of sample buffer containing a reducing agent (5-20mM DTT final concentration). Protein lysates were resolved by SDS PAGE (10% acrylamide) under reducing conditions and transferred to nitrocellulose membranes (Schleicher and Schuell). The protein bands were visualized with Ponceau S (Sigma). Membranes were blocked with 4% non-fat dried milk in TBST (20 mM Tris-Hcl, 150 mM NaCl, 0.1% Tween 20) supplemented with 1% bovine serum albumin (BSA). Blots were then incubated at room temperature for 1 h with primary antibody diluted in TBST/1%BSA. Horseradish peroxidase-conjugated secondary antibodies were used to visualize bound primary antibodies with the Supersignal chemiluminescence substrate (Pierce).

**Peptide Competition.** CHO cells were transiently transfected with cDNA encoding the long isoform and subjected to preparative SDS-PAGE gel. After transfer, the nitrocellulose membrane was cut into strips and incubated with anti-ATR/TEM8 polyclonal antibody alone or in combination with peptides. Horseradish peroxidase-
conjugated secondary antibodies were used to visualize bound primary antibodies with the Supersignal chemiluminescence substrate (Pierce).

**Immunohistochemistry.** Paraffin sections were de-paraffinized in xylene (twice, 10 min each), hydrated through a graded series of ethanol washes and placed in PBS. Antigen retrieval was performed by heating at 95°C for 15 min in citrate buffer. Endogenous peroxide activity was quenched by incubation with the peroxidase blocking reagent (DAKO). Sections were incubated over-night with the anti-ATR/TEM8 polyclonal antibody. After washing, the slides were incubated with a biotin-conjugated secondary antibody. Then, slides were incubated with horseradish-peroxidase-conjugated streptavidine (DAKO). Bound antibodies were visualized using DAB as the substrate. Then, sections were counterstained with hematoxylin, and dehydrated. The slides were mounted with a xylene-based mounting media (Micromount, Surgipath) and observed with an Olympus IX 70 inverted microscope.
Results and Discussion

A cellular receptor for PA was recently identified as anthrax toxin receptor (ATR), a transmembrane protein that results from alternative splicing of the tumor endothelial marker 8 (TEM8) gene. The physiological function of ATR/TEM8 has not been yet fully elucidated, but several lines of evidences suggest that ATR/TEM8 may play a role in angiogenesis. For example, TEM8 transcript levels were discovered to be selectively elevated in colorectal cancer endothelium (19, 24). In addition, ATR/TEM8 is abundantly expressed in tumor-associated blood vessels as well as in the vasculature of developing murine embryos (19, 24). In contrast, anthrax toxin receptor "activity" has been localized to the basolateral surface of polarized epithelial cells (3). As such, the cellular localization and the tissue distribution of the ATR/TEM8 protein remains controversial.

In this report, we attempt to elucidate the distribution of ATR/TEM8 in tissues that constitute the primary targets for the anthrax infection. For this purpose, we generated and characterized a novel anti-ATR/TEM8 polyclonal antibody that selectively recognizes all thee ATR/TEM8 isoforms (long, medium, and short).

A novel anti-ATR/TEM8 polyclonal antibody detects all three ATR/TEM8 isoforms.

Figure 1A shows a schematic diagram of the three ATR/TEM8 isoforms resulting from alternative splicing of the TEM8 gene. The long isoform (TEM8) contains 564 amino acids with a transmembrane region and a long proline-rich cytoplasmic tail. The medium isoform (ATR) encodes a 368-amino-acid protein with a transmembrane region and a short cytoplasmic tail. The long and medium isoforms are identical throughout the extracellular and transmembrane domains, but diverge at the last four amino acids of the cytoplasmic tail at the C-terminus. As such, the medium and long isoforms both function
as receptors for the anthrax toxin (6). On the contrary, the short isoform encodes a 333 amino acids protein, which is identical to the other two isoforms in the extracellular domain, but profoundly diverges just before the putative transmembrane domain, and is lacking any sequence for membrane attachment. As a consequence, the short putative secreted isoform does not function as a receptor for the anthrax toxin.

All three ATR/TEM8-isoforms share a common extracellular von Willebrand factor type A (VWA) domain (residues 44-216). To generate a novel rabbit polyclonal antibody directed against ATR/TEM8, we used a sequence within the VWA domain of ATR/TEM8 (residues 92-107; LMKLTEDREQIRQGLE) (Figure 1A). Importantly, we chose this ATR-epitope because is completely divergent from an ATR-related protein, namely CMG2 (ILPLTGDRGKISKGLE), at this position; note that 8 out of the 16 amino acid residues are not conserved.

An alignment of the C-terminal domain sequences of the three isoforms illustrates that the three isoforms profoundly diverge at the C-terminus (Figure 1B). Note that the short isoform lacks a transmembrane domain, while the medium isoform has a 25 amino acid cytoplasmic tail, as compared to the 221 amino acid cytoplasmic tail of the long isoform.

To test the immunoreactivity of our novel anti-ATR/TEM8 antibody, we transiently transfected CHO cells with Myc-tagged cDNAs encoding the short, medium, and long ATR/TEM8 isoforms. Western blot analysis revealed that the ATR/TEM8 antibody successfully recognizes all three isoforms (Figure 2A). Importantly, the different isoforms can be distinguished based on their respective molecular weight. The short isoform is a 45 kDa protein, the medium isoform is a ~ 60 kDa protein, and the long isoform is an ~ 80-85 kDa doublet. This doublet is probably due to multiple glycosylation events. Interestingly, this doublet was also detected in untransfected CHO
cells, suggesting that the ATR/TEM8 antibody recognizes the long endogenous isoform. To independently verify these results, we used three isoforms containing a Myc-tag at the C-terminal, and performed immunoblotting with an anti-Myc polyclonal antibody. Figure 2B shows that all three isoforms are strongly expressed in CHO cells. These results demonstrate the specificity and the strong affinity of the novel anti-ATR/TEM8 polyclonal antibody, as it detects exogenous as well as endogenous ATR/TEM8 isoforms.

The anti-ATR/TEM8 antibody is sensitive to peptide competition with the immunogen.

We further analyzed the specificity of the anti-ATR/TEM8 antibody by competition with the peptide used for antibody production (the immunogen) and with an irrelevant peptide. CHO cells were transiently transfected with the cDNA encoding the long isoform. As expected, Western blot analysis with the anti-ATR/TEM8 antibody detected an ~80-85 kDa doublet corresponding to the long endogenous isoform (Figure 3). However, immunoreactivity was abolished by preincubation of the anti-ATR/TEM8 antibody with a 100-molar excess of the antigenic peptide (Figure 3). Importantly, the immunoreactivity of the antibody was not affected by an irrelevant peptide (Figure 3). These results demonstrate the highly selectivity of the novel ATR/TEM8 antibody in detecting ATR/TEM8 isoforms.

ATR/TEM8 isoforms are widely expressed in a variety of mouse tissues.

In order to gain insight into the pathogenesis of anthrax infection, we next examined the tissue distribution of the anthrax toxin receptor (ATR/TEM8). We performed Western Blot analysis on a variety of mouse tissue lysates. Figure 4 shows that the ATR/TEM8 antibody successfully detects the endogenous expression of all three ATR/TEM8 isoforms, distinguishable by their different molecular weights. We found that the ATR/TEM8 isoforms are widely and differentially expressed. The long isoform is
predominantly expressed in the spleen, lung, and kidney, while the medium isoform is mainly expressed in the liver and kidney. Finally, the short isoform is particularly enriched in the heart, brain, and kidney (summarized in Table 1).

These results are in conflict with previous findings, in which the TEM8 distribution on human and mouse tissue was evaluated by in situ hybridization (9, 24), and by immunohistochemistry (19). Those studies showed that TEM8 expression is restricted to tumor endothelium and to developing vasculature, and undetectable in normal tissues.

**ATR/TEM8** is highly expressed in epithelial cells lining the toxin’s three sites of entry: Lung, skin, and small intestine.

Next, we attempted to evaluate the detailed tissue distribution of ATR/TEM8 by immunohistochemistry. We tested the ATR/TEM8 expression in the three tissues that represent the main route of entry of the anthrax toxin, i.e. lung, skin, and intestine. Since the pulmonary form is the most lethal form of the anthrax disease, we first assessed ATR/TEM8 expression on paraffin-embedded sections from mouse lung. Surprisingly, ATR/TEM8 expression was strongly detectable in the respiratory epithelium of the bronchi of the lung (Figure 5). In particular, an intense staining was evident in the ciliated epithelial cells surrounding the luminal surface. In addition, ATR/TEM8 was also expressed in the smooth muscle cells surrounding the vessels, as well as in epithelial cells lining the alveoli. We observed a weak or undetectable staining in the endothelial cells lining the pulmonary vessels (Figure 5). Taken together, the tissue distribution of ATR/TEM8, as determined by immunohistochemistry with the anti-ATR/TEM8 polyclonal antibody, correlated with the primary uptake sites of the anthrax toxin.

To evaluate whether the ATR/TEM8 epithelial localization is mouse-specific or a
more general phenomenon, we performed immunohistochemistry with the ATR/TEM8 polyclonal antibody on human lung paraffin-embedded sections. Figure 6 shows that ATR/TEM8 staining is concentrated in the respiratory epithelium of the bronchi in the lung. In particular, the ciliated epithelial cells lining the bronchi display strong ATR/TEM8 expression. Taken together, these results suggest that normal respiratory epithelial cells can effectively bind and internalize more molecules of anthrax toxin than other cell types, causing the lethal pulmonary form of the disease.

Microscopic or gross breaks of the skin represent another important route for anthrax infection. For this reason, we evaluated ATR/TEM8 expression in skin biopsies. Immunohistochemistry with the ATR/TEM8 polyclonal antibody on mouse skin paraffin-embedded sections revealed that ATR/TEM8 is strongly expressed in the epithelial keratinocytes of the epidermis (Figure 7). To verify the epithelial localization of the ATR/TEM8 in other species, we also analyzed the ATR/TEM8 expression in humans. Figure 8 shows that ATR/TEM8 display a similar expression pattern in both mouse and human skin. ATR/TEM8 staining is particularly intense in the thick layer of epithelial cells of the epidermis.

The intestine represents the third main route of infection of the anthrax toxin. As such, we attempted to evaluate the expression pattern of ATR/TEM8 in the small intestine. Interestingly, immunohistochemistry with the ATR/TEM8 polyclonal antibody on mouse small intestine paraffin-embedded sections revealed that ATR/TEM8 is highly expressed in the epithelial cells lining the intestinal crypts (Figure 9).

**ATR/TEM8 isoform expression in lung, skin, and small intestine.**

We next performed Western blot analysis on lysates prepared from normal mouse lung, skin, and small intestine (Figure 10). Interestingly, the long isoform is
predominantly expressed in the lung and in the small intestine, but is still present at lower levels in skin. In contrast, the medium isoform is absent from the lung, but is highly expressed in the small intestine, and to a lesser extent in the skin. Finally, the short isoform is present only in skin (summarized in Table 1). Thus, these three sites of entry also differentially express all three ATR/TEM8 isoforms—in a tissue-specific fashion.

Conclusions

Taken together, our results clearly show that the receptor for the anthrax toxin is highly expressed in the epithelial cells lining organs that represent the major uptake routes of the toxin. These observations can explain the increased sensitivity of certain organs, i.e. lung, skin, and intestine, to anthrax infection. They suggest that the infection route may be dependent on the abundant expression of the cellular receptor in epithelial cells. As such, these findings provide new important clues for understanding the function of ATR/TEM8, and the mechanisms that underlie the pathogenesis of anthrax infection.
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References

15. Lacy DB, Wigelsworth DJ, Scobie HM, Young JA, and Collier RJ. Crystal structure of the von Willebrand factor A domain of human capillary morphogenesis
Figure Legends

Figure 1. Schematic representation of the ATR/TEM8 isoforms and epitopes used for antibody production.

(A) The three different ATR/TEM8 isoforms, resulting from alternatively spliced TEM8 mRNA transcripts, are shown. The long isoform contains 564 amino acids, the medium isoform 368 amino acid, and the short protein 333 amino acids. A rectangular box indicates a sequence within the VWA extracellular domain that is conserved in all three ATR/TEM8 isoforms. This sequence (LMKLTEDREQIRQGLE, residues 92-107) corresponds to the peptide used to generate a novel anti-ATR/TEM8 polyclonal antibody.

(B) Alignment of the cytoplasmic C-terminal sequences of the three isoforms. A box indicates the conserved residues in the putative transmembrane domain (TM, residues 321-343) of the long and medium isoforms. As the short isoform lacks the putative transmembrane domain, it is predicted to be secreted. Note that the cytoplasmic C-terminal domains of the medium and long isoforms are divergent, such that cytoplasmic tail of medium isoform contains only 25 amino acids whereas the cytoplasmic tail of the long isoform contains 221 amino acids.
Figure 2. A novel anti-ATR/TEM8 polyclonal antibody detects all three ATR/TEM8 isoforms.

(A) CHO cells were transiently transfected with the cDNAs encoding for the short, medium, and long isoforms of ATR/TEM8. Immunobloting with the ATR/TEM8 polyclonal antibody reveals that this new probe successfully recognizes all three isoforms. Note that the ATR/TEM8 antibody identifies the long isoforms as an ~80-85 kDa doublet, probably due to multiple glycosylation events. Interestingly, the doublet is also detected, albeit at lower levels, in CHO cells transfected with the short and medium isoforms, as well as in untransfected cells. These results indicate that the novel anti-ATR/TEM8 polyclonal antibody also detects the endogenous long isoform of the receptor.

(B) CHO cells were transiently transfected with the cDNAs encoding the Myc-tagged short, medium, and long ATR/TEM8 isoforms. Immunoblotting with an anti-Myc polyclonal antibody reveals that all three isoforms are properly expressed. No ATR/TEM8 expression is detectable in the untransfected cells.
Figure 3. The anti-ATR/TEM8 antibody is sensitive to peptide competition with the immunogen.

CHO cells were transiently transfected with the cDNA encoding the long ATR/TEM8 isoform, and subjected separation using a preparative SDS-PAGE gel. After transfer, the nitrocellulose membrane was cut into strips and incubated with the anti-ATR/TEM8 polyclonal antibody alone or in combination with nonspecific or ATR/TEM8-specific peptides. As expected, the anti-ATR/TEM8 antibody alone recognizes the long isoform as an ~80-85 kDa doublet. Note that, after preincubation with the peptide antigen used for the antibody production (the immunogen), the anti-ATR/TEM8 antibody no longer recognizes the ~80-85 kDa doublet. In contrast, preincubation of anti-ATR/TEM8 IgG with an irrelevant peptide does not affect the immuno-reactivity of the antibody.
Figure 4. **ATR/TEM8 isoforms are widely expressed in a variety of mouse tissues.**

Western blot analysis with the anti-ATR/TEM8 polyclonal antibody was performed on a variety of mouse tissues lysates. The three ATR/TEM8 isoforms are differently expressed. The long isoform is predominantly expressed in spleen, lung, and kidney. In contrast, the medium isoform is mainly expressed in liver and kidney, and the short isoform is preferentially expressed in heart, brain, and kidney. Both a short (Upper Panel) and a longer exposure (Lower Panel) are shown.
Figure 5. **ATR/TEM8 is highly expressed in the epithelial cells of murine lung.**

Paraffin-embedded sections of mouse lung were subjected to immunohistochemistry with anti-ATR/TEM8 polyclonal antibody, and counterstained with hematoxylin. ATR/TEM8 is strongly expressed in the respiratory epithelium of the bronchi (b) of the lung. Intense staining is evident in the ciliated cells surrounding the lumen of the bronchial tube (Panels 3 and 4). The endothelial cells lining the vessels (v) show weak or undetectable staining (Panel 1, 2, and 4), whereas the smooth muscle cells surrounding the vessels (v) highly express the ATR/TEM8 proteins (Panel 1). A higher magnification view is shown to better illustrate this point (panel 5). b, bronchi; v, vessel.
Figure 6. Epithelial cells of the human lung express high levels of ATR/TEM8.

Immunohistochemistry with the anti-ATR/TEM8 polyclonal antibody was performed on human lung paraffin-embedded sections. ATR/TEM8 is highly expressed in the respiratory epithelium of the bronchi (panel 1). Intense ATR/TEM8 staining occurs in the ciliated epithelial cells lining the lumen of the bronchial tube (panel 2).
Figure 7. **ATR/TEM8 is highly expressed in the epithelial cells (keratinocytes and follicular cells) of murine skin.**

Immunohistochemistry with the anti-ATR/TEM8 polyclonal antibody was performed on murine skin paraffin-embedded sections. Panel 1 reveals strong ATR/TEM8 expression in the thin epithelium of the epidermis, as well in the hair follicles. Panel 2 shows a section of panel 1 at higher magnification.
Figure 8. **ATR/TEM8 is highly expressed in the epithelial cells of human skin.**

Immunohistochemistry with the anti-ATR/TEM8 polyclonal antibody was performed on paraffin-embedded sections of human skin. Robust ATR/TEM8 expression is evident in the thick epithelium of the epidermis (panel 1). Panel 2 shows a section of panel 1 at higher magnification.
Figure 9. ATR/TEM8 is highly expressed in the epithelial cells of mouse small intestine.

Immunohistochemistry with the anti-ATR/TEM8 polyclonal antibody was performed on paraffin-embedded sections of mouse small intestine. Note that ATR/TEM8 is strongly expressed in the epithelial cells lining the intestinal crypts. A higher magnification is shown to better illustrate this point (panel 2).
Figure 10. A comparison of ATR/TEM8 isoforms in lung, skin, and small intestine.

Western blot analysis, using anti-ATR/TEM8 IgG, was performed on lysates prepared from normal mouse lung, skin, and small intestine. Note the different levels of expression of the three ATR/TEM8 isoforms. The long isoform is predominantly expressed in the lung and in the small intestine. In contrast, the medium isoform is absent from the lung, but is highly expressed in the small intestine, and to a lesser extent in the skin. Finally, the short isoform is present only in skin.
ATR/TEM8 Isoforms

Epitope

LMKLTEDREQIRQGLE

Long:
92-107

Medium:
321-343
368

Short:
321-343
333

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Figure 1A
C-terminal Sequences of ATR/TEM8 Isoforms

Transmembrane Domain

| 317 | CSGSILAIALLILFLLLLALALLLWWFWPLCCTVIIEVEVPPPAEESEEE | Long |
| 333 | CSLHKIASGPTTAACME | Medium |
| 368 | KIK | Short |

| 357 | DDDGLPKKKWPTVDASYYGGVGIGKRMKVWGEKGGSTEEGAKLEAK | Long |
| 368 | KIK | Medium |

| 368 | NARVHMPEQEFPEPRNLNNMRRPSSPKWSPIKGLDAWLNRK | Long |

| 368 | GYDRSVSVMQQPQPDGTCRINFTVKNQPAKYPLNNAYHTSSPPAPIY | Long |

| 564 | TPPPAPHCPPPPSSAPTPPIPSPPFLPPPQAPPNPNPSPRPPPR | Long |

| 564 | PSV | Long |
Bonuccelli et al., Figure 2

A

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ATR/TEM8 pAb

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Myc pAb
Peptide Competition

Bonuccelli et al., Figure 3
Tissue Distribution

Short Exposure:

- Long form
- Medium form
- Short form

Bonuccelli et al.,
Figure 4

Longer Exposure:

- Long form
- Medium form
- Short form
Normal Mouse Lung

ATR/TEM8 Immuno-staining

Bonuccelli et al., Figure 5
Bonuccelli et al.,
Figure 5 (continued)
Normal Human Lung

ATR/TEM8 Immuno-staining

Bonuccelli et al., Figure 6
Normal Mouse Skin

ATR/TEM8 Immuno-staining
Normal Human Skin

ATR/TEM8 Immuno-staining
Normal Mouse Intestine
ATR/TEM8 Immuno-staining

Bonuccelli et al.,
Figure 9
ATR/TEM8 Isoform Expression

Bonuccelli et al.,
Figure 10

[Image of a Western blot showing isoform expression in lung, skin, and small intestine]
**Table 1.** Summary of the Tissue-Specific Expression of ATR/TEM8 Isoforms.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>ATR Isoform Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>Short &gt;&gt; Long</td>
</tr>
<tr>
<td>Ovary</td>
<td>Long</td>
</tr>
<tr>
<td>Testis</td>
<td>Long</td>
</tr>
<tr>
<td>Spleen</td>
<td>Long</td>
</tr>
<tr>
<td>Liver</td>
<td>Medium &gt;&gt; Short &amp; Long</td>
</tr>
<tr>
<td>Brain</td>
<td>Short</td>
</tr>
<tr>
<td>Kidney</td>
<td>Long, Medium, &amp; Short</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>Long</td>
</tr>
<tr>
<td>Lung&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Long</td>
</tr>
<tr>
<td>Skin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Long, Medium, &amp; Short</td>
</tr>
<tr>
<td>Small Intestine&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Long &amp; Medium</td>
</tr>
</tbody>
</table>

<sup>a</sup> bronchiolar epithelial cells and smooth muscle cells;  <sup>b</sup>, keratinocytes (especially the basal cell layer) and hair follicle cells;  <sup>c</sup>, intestinal epithelial cells (especially within the crypts).