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ATF6 and Thrombospondin 4: The Dynamic Duo of the Adaptive Endoplasmic Reticulum Stress Response

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Summary

Thrombospondins are secreted, extracellular matrix (ECM) proteins that are upregulated in the heart and other tissues in response to ischemic injury and myocardial stress. Roles for thrombospondins after they are secreted have been examined in a variety of disease models, including myocardial pathology. However, a recent study published in the journal *Cell* by Lynch et al[1] shifts this paradigm by focusing on roles for intracellular thrombospondins; these authors showed that thrombospondin-4 (Thbs4) can function from within cells to protect the heart by enhancing adaptive aspects of the endoplasmic reticulum (ER) stress response that are mediated by activating transcription factor 6, ATF6. Although this study was carried out in the cardiac context, the results add to our understanding of protein folding and quality control in all tissues. Moreover, the findings underscore the potential widespread therapeutic benefit of enhancing adaptive responses that are regulated by ATF6.

Keywords

thrombospondin-4; ATF6; GRP78; ER stress; unfolded protein; cardioprotection

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The study by Lynch et al[1], which was carried out in the laboratory of Dr Jeffery Molkentin, employed a series of molecular approaches, including *in vivo* gain- and loss-of-function models in mice to demonstrate new roles for Thbs4 in the management of ER protein quality control in the heart. The ER is the site of synthesis and folding of most secreted and membrane proteins, which constitute at least 35% of all proteins[2]. Secreted and membrane proteins are synthesized by ER-bound ribosomes, co-translationally translocated across the ER membrane, and folded in the lumen of the ER, after which they are transported to the Golgi, where they are sorted to their final destinations[3, 4]. The ER lumen is populated with chaperones, protein disulfide isomerases, protein oxidoreductases, and other proteins that are responsible for the proper folding of secreted and membrane proteins. This elaborate machinery requires an optimal ER environment for efficient protein folding. Conditions such as myocardial ischemia, hypertrophy, and heart failure alter this environment in ways that reduce protein folding, leading to the accumulation of misfolded, potentially toxic[5] proteins that can cause ER stress[6].

The ER stress response averts the potential proteotoxicity associated with the accumulation of misfolded proteins in the ER during ER stress[7-9]. Three ER transmembrane proteins, PERK (protein kinase RNA-like ER kinase), IRE-1 (inositol-requiring protein-1), and ATF6 (activating transcription factor 6) serve as sensors of misfolded proteins in the ER as well as effectors of the response to ER stress (Fig. A). Although the mechanisms by which these

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sensors detect misfolded proteins are not completely understood, one of the first mechanisms to be described involves the ubiquitous ER-luminal chaperone, glucose regulated protein 78 kD (GRP78), which plays numerous roles in ER protein folding and quality control[10, 11]. When protein folding in the ER is optimal, GRP78, which has a C-terminal ER retention motif, or KDEL, binds to the ER-luminal domains of the three proximal sensors and keeps them inactive[12, 13] (Fig. A). Upon ER stress, GRP78 relocates from the proximal sensors to misfolded proteins in efforts to fold them (Fig. B). This relocation of GRP78 has different effects on the three sensors. In the case of ATF6, which is a major topic of the study by Lynch et al, the relocation of GRP78 releases ATF6 from the ER, permitting its transport to the Golgi (Fig. C), which is facilitated by Golgi localization signals[13]. In the Golgi, Site-1 and Site-2 proteases (S1P and S2P) cleave the trans-ER membrane region of ATF6 (Fig. D). This liberates the N-terminal, cytosolic domain of ATF6, which translocates to the nucleus, where it binds to and induces nearly 400 genes in the myocardium[14] (Fig. E); many of these genes are known to contribute to restoring ER protein folding capacitymuy and enhancing cardioprotection[15] via pleiotropic adaptive responses[14] (Fig. F). Thus, the conditional interaction of ATF6 with the ER-resident GRP78 is a determinant of ATF6 location and, thus, ATF6 activity. Lynch et al found that ATF6 location and activity can also be determined by its interaction with Thbs4.

The thrombospondin (Thbs) family is composed of five members (Thbs1-5) whose expression and secretion from numerous cell types, including cardiac myocytes, is increased during pathology and tissue damage[16]. Thrombospondins form two subfamilies. Thrombospondins 1 and 2 assemble as homo- and heterotrimers, while the remaining Thsb family members form homo- or heteropentamers. Most studies have focused on the functions of Thbs after its secretion, upon which it interacts with various structural ECM proteins and contributes to matrix remodeling during the course of pathology and response to tissue injury. Under most conditions, Thbs is expressed at low levels in cardiac myocytes, but is expressed at moderate levels in other cell types in the heart [17]. Thbs1 and Thbs2 knockout mouse studies have shown that these thrombospondins protect against myocardial infarction (MI) injury and overload-induced hypertrophy[18]. Thbs4 has captured recent attention because it is expressed primarily in cardiac and skeletal muscle, and is further upregulated upon myocardial infarction, hypertrophy, and heart failure[19, 20].

The synthesis and assembly of Thbs4 has not been studied in detail; however, by analogy to studies on other Thbs family members[17, 21, 22], it can be inferred that Thbs4 monomers (~900 AA, ~100 kD each), which have no transmembrane motifs, are synthesized on ER-bound ribosomes, followed by folding and assembly into hetero- or homopentameric oligomers in the ER lumen (Fig. G; shown as homopentamers). Since it does not have a C-terminal KDEL motif to facilitate ER retention, Thbs4 presumably travels rapidly from the ER to the Golgi (Fig. H) on its way to secretion via the constitutive secretory pathway (Fig. I), a process by which most secreted proteins are released from cells at rates dictated primarily by their expression levels[3, 23]. Although Thbs secretion from cardiac myocytes has not been studied in detail, Thbs has been shown to be released from endothelial cells within 60 min of its synthesis[21], consistent with rapid, constitutive secretion. To fold properly thrombospondins must be glycosylated. In endothelial cells, the impairment of protein glycosylation in the ER by tunicamycin leads to Thbs misfolding, which results in its retention in the ER and inhibition of secretion[21]. After its secretion, Thbs contributes to cardioprotection through processes such as adaptive extracellular matrix remodeling (Fig. J).

Targeted disruption of Thbs4 in the mouse heart has been shown to increase the maladaptive effects of pressure overload; these effects were attributed to extracellular Thbs4[18, 24, 25]. However, the study by Lynch et al represents a paradigm shift, because it identifies a new function for intracellular Thbs4, linking it to ATF6-mediated cardioprotection. To determine

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the role of Thbs4 in the heart, Lynch et al overexpressed Thbs4 in mouse hearts and showed that it enhanced survival and preserved cardiac function after MI. The effects of pressure overload were unclear, since there was no apparent loss of cardiac function in either control or Thbs4 transgenic mice subjected to trans-aortic constriction. The authors performed a gene expression analysis to gain further insight into the mechanism of Thbs4-mediated cardioprotection; although the data were not shown, the authors concluded that many genes previously shown to be increased in the hearts of transgenic mice expressing activated ATF6 in cardiac myocytes[14] were also upregulated in the hearts of Thbs4 transgenic mice. The authors showed that Thbs4 increased the levels of cleaved ATF6, as well as numerous ATF6-inducible proteins. This finding led to the authors' hypothesis that Thbs4 might exert at least some of its protective effects by facilitating ATF6 activation. In support of this hypothesis, the authors showed that Thbs4 interacted with ATF6. To examine whether Thbs4-mediated activation of ATF6 required translocation to the Golgi, they generated a form of Thbs4 with a C-terminal ER retention KDEL motif (Thbs- KDEL), which should convert Thbs4 into an ER resident protein. Unlike native Thbs4, Thbs4-KDEL did not increase the levels of activated ATF6, presumably because it resided in the ER and was thus unable to facilitate ATF6 transport to the Golgi. The study also showed that the ATF6 activation observed in the hearts of wild-type mice subjected to overload-induced hypertrophy and MI was lost in Thbs4 knockout mouse hearts. Moreover, compared to wild-type mice, the hearts of the Thbs4 knockout mice exhibited reduced cardiac function and increased injury in the hypertrophy and MI models, respectively. These results are consistent with the hypothesis that Thbs4 mediates protection from cardiac pathology, at least partly by activating ATF6 and the subsequent expression of genes downstream of ATF6.

The results of this study have broad impact because they suggest that, in addition to its roles in ECM remodeling after secretion, Thbs4 has cardioprotective functions before its secretion. Moreover, since Lynch et al showed that Thbs1, which is in a different subfamily, and structurally somewhat different than Thbs4, also facilitated ATF6 activation, it is apparent that other members of the Thbs family activate ATF6. In terms of determining the relative importance of intra- and extracellular Thbs, Lynch et al showed that, in contrast to overexpressed Thbs4, the addition of recombinant Thbs4 to culture media did not increase activated ATF6, consistent with the idea that only intracellular Thbs4 can bind to and activate ATF6. However, the relative contributions of intra- and extracellular Thbs4 in the cardioprotection that was observed *in vivo* remain unknown.

As a liberator of ATF6 from the ER, Thbs4 is a counterpart of GRP78, which anchors ATF6 in the ER. The mechanism responsible for these opposing actions is likely to be complex and will require further study to delineate. However, coupled with previous studies, the study by Lynch et al supports several different mechanisms. For example, since overexpressing Thbs4 activates ATF6, and since ATF6 activation requires its liberation from the ER, then overexpressed Thbs4 must liberate ATF6 from the ER by overcoming the ability of GRP78 to anchor ATF6 in the ER. This could happen if Thbs4 is able to physically displace GRP78 from ATF6. The relatively close proximity of the GRP78 binding sites on ATF6, which were described previously[13], to the Tbhs4 binding sites on ATF6, described by Lynch et al, supports this hypothesis, leading to the concept that the winner of the competition serves as a determinant of ATF6 location. Moreover, the site on ATF6 to which Lynch et al showed Thbs4 binds was previously shown to serve as a Golgi localization sequence, which is masked when GRP78 binds to ATF6[13]. This is consistent with the hypothesis that GRP78 dislocation from ATF6 unmasks a Golgi localization sequence to which Thbs4 can bind (Fig. K), which then facilitates ATF6 translocation to the Golgi (Fig. L). Thus, the relative amounts of GRP78 and Thbs4 could determine whether ATF6 remains in the ER or relocates to the Golgi. Furthermore, it is possible that Thbs4 overexpression may additionally contribute to ATF6 translocation to the Golgi by attracting

GRP78 away from ATF6 to assist in the folding of nascent Thbs4. In support of this possibility are previous studies showing that GRP78 binds to Thbs while it is being synthesized in the ER[26, 27].

A provocative result from this study was the finding that overexpressed Thbs4 increased the secretion of both atrial natriuretic factor (ANF) and mesencephalic astrocyte-derived neurotrophic factor (MANF)[1] from neonatal cardiac myocytes. The authors suggested that this may be due to the ability of Thbs4 to enhance the secretory capacity of the ER. While this may be a contributing factor, the ability of Thbs4 to coordinately increase the secretion of both of these proteins is unexpected, since ANF and MANF are secreted under different conditions and via different mechanisms. In the case of ANF, since its secretion from ventricular myocytes is constitutive, and therefore dictated primarily by its expression levels, increased levels of secreted ANF are a reflection of increased cellular levels of ANF[28]. Thus, perhaps Thbs4 overexpression increased cellular ANF, which resulted in increased secretion. In contrast to ANF, MANF secretion from ventricular myocytes is not constitutive because, like ATF6, MANF binds to GRP78, which conditionally retains MANF in the ER. Disruption of MANF binding to GRP78 has been shown to release MANF from the ER and, since it has no transmembrane domains, MANF is secreted[29]. Similar to ATF6, it is the conditional association of MANF with GRP78 that dictates its location. Accordingly, it may be that Thbs4 increases MANF secretion by liberating it from the ER-anchoring effects of GRP78, much like it does with ATF6.

The study by Lynch et al did not address whether Thbs4 is required for the activation of ATF6 during ER stress. However, previous studies have shown that when ER stress is activated, either by inhibiting protein glycosylation with tunicamycin, or by decreasing ER calcium, which are conditions under which ATF6 translocates from the ER to the Golgi where it is cleaved and activated, Thbs exit from the ER and secretion are blocked[21, 30] and Thbs is degraded. Accordingly, at least under these conditions of ER stress, the binding of Thbs4 to ATF6 may not be required for ATF6 translocation and activation (Fig. L). Thbs4-mediated activation of ATF6 in the absence of ER stress is consistent with the hypothesis that ATF6 may have functions beyond its known roles as a first responder to ER protein misfolding[31, 32]. In support of this hypothesis is a study by Wu et al[33] which showed that ATF6 was activated during physiological skeletal muscle exercise, and that this activation was required for metabolic adaptation to exercise training. This supports roles for ATF6-mediated adaptive responses beyond those that take place upon acute ER protein misfolding. Perhaps, like it does in skeletal muscle, ATF6 contributes to adaptive responses to physiological conditions in cardiac muscle that are not necessarily associated with overt activation of ER stress. Moreover, the findings reported by this study raise awareness to the potentially beneficial effects of Thbs4 and ATF6 in treating a vast array of pathologies associated with protein misfolding in the ER.

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[1]MANF was called ARMET (arginine-rich, mutated in early stage tumors) by Lynch et al.

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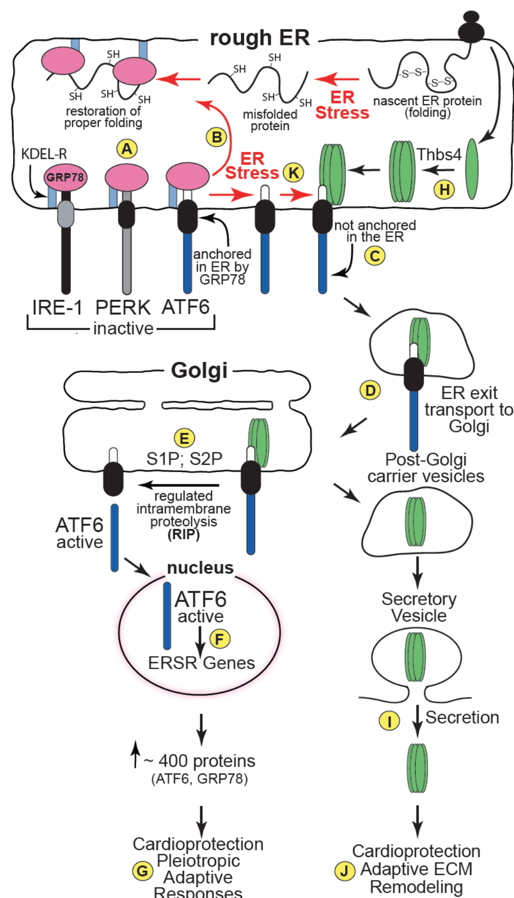

Figure. Roles of GRP78 and Thrombospondin-4 in ATF6 Activation

The ER-transmembrane proteins, IRE-1, PERK, and ATF6, are sensors of protein folding in the ER. When ER protein folding is optimal, the ER luminal domains of each sensor are associated with the ER chaperone, GRP78 **(A)**. GRP78 is an ER resident protein by virtue of its C-terminal KDEL sequence, which facilitates its retrieval from the Golgi to the ER via its binding to the KDEL receptor. Because it binds to GRP78, ATF6 is anchored in the ER, which keeps ATF6 from being activated. However, when ER protein folding is impaired, for example, during ER stress (red arrows), GRP78 relocates to misfolded proteins as they accumulate **(B)**; since, under these conditions, GRP78 is no longer bound to ATF6 in the ER, ATF6 is free to relocate to the Golgi **(C)**. In the Golgi, ATF6 is cleaved by S1P and S2P **(D)** to form an active transcription factor that induces ER stress response genes **(E)**, including ATF6 and GRP78, which primarily mediate pleiotropic adaptive, or protective responses **(F)**. Thrombospondin-4 (Thbs4) is synthesized as monomers in the ER, which assemble into pentamers **(G)** that transit from the ER lumen to the Golgi **(H)**, then to secretory vesicles, from which Thbs4 is eventually secreted **(I)**. Extracellular Thbs4 binds to structural ECM proteins and modulates the ECM in response to tissue damage, providing cardioprotective and adaptive ECM remodeling **(J)**. The study by Lynch et al showed that Thbs4 can bind to ATF6 **(K)** and facilitate its activation, most likely by facilitating its translocation to the Golgi **(L)**. Since Thbs4 is retained in the ER during some ER stress treatments that are known to stimulate ATF6 translocation to the Golgi (see text), it may be that there are some conditions under which ATF6 relocation from the ER to the Golgi, and thus, ATF6 activation can take place in a Thbs4-independent manner **(C and D)**.