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Regulation of expression of *CPB2*, the gene encoding human thrombin activatable fibrinolysis inhibitor (TAFI): the role for post-transcriptional regulation

By

Dragana Komnenov

A Dissertation
Submitted to the Faculty of Graduate Studies
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy
at the University of Windsor

Windsor, Ontario, Canada

September, 2014

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Regulation of expression of *CPB2*, the gene encoding human thrombin activatable fibrinolysis inhibitor (TAFI): the role for post-transcriptional regulation

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Declaration of Co-Authorship / Previous Publication

I. Co-Authorship Declaration

I hereby declare that this thesis incorporates material that is result of joint research, as follows:
This thesis also incorporates the outcome of a joint research undertaken in collaboration with Andrew C.Y. Kuo and Joellen H.Lin under the supervision of Dr. Michael B. Boffa. The collaboration is covered in Chapter 2 of the thesis. Primary contributions, experimental designs, data analysis and interpretation, were performed by the author, and the contribution of co-authors was primarily through the collection of data for Figures 1-6.

II. Declaration of Previous Publication

This thesis includes 2 original papers that have been previously published/submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication title/full citation	Publication status
Chapter 2	Identification of tristetraprolin as a factor that modulates the stability of the TAFI transcript through binding to the 3'-untranslated region	published
Chapter 3	Inflammatory cytokines reduce thrombin activatable fibrinolysis inhibitor (TAFI) expression via tristetraprolin-mediated mRNA destabilization	submitted

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Abstract

Disequilibrium between coagulation and fibrinolysis can lead to severe haemostatic disorders such as thrombosis and hemophilia. Thrombin-activable fibrinolysis inhibitor (TAFI) is a carboxypeptidase B-like pro-enzyme that, once activated, attenuates fibrinolysis. TAFI may also mediate connections between coagulation and inflammation. Studies have associated high plasma TAFI levels with a risk for thrombotic diseases. TAFI plasma concentrations vary substantially within human population, and various hormonal factors and disease states have been shown to have an impact. Regulation of expression of the gene encoding TAFI, *CBP2*, is likely an important determinant of the role of the TAFI pathway *in vivo*; this concept motivated the investigations described in this thesis.

Our first set of studies lead to the identification of key *cis*-acting sequences within the 3'-untranslated region (3'-UTR) of the TAFI mRNA that specify transcript stability. Specifically, we described the presence of one stability element, followed by three instability elements. Furthermore, we identified the *trans*-acting factor binding to the last instability element. Tristetraprolin (TTP) is capable of binding this sequence, promoting mRNA destabilization and degradation. We also observed that another *trans*-acting protein factor, HuR, binds the TAFI 3'-UTR.

We found that TTP and HuR play a crucial role in post-transcriptional regulation of *CPB2* transcript. Pro-inflammatory mediators exerted their TAFI protein-lowering effects via TTP-mediated mRNA destabilization in human hepatocellular carcinoma (HepG2) cells. On the other hand, *CPB2* mRNA and TAFI protein abundance and transcript stability were increased in THP-1 macrophages in the presence of inflammatory

mediators, suggesting the possibility of tissue-specific regulation for *CPB2* gene expression.

We also obtained preliminary evidence that miR-124, miR-506 and miR-708 decrease endogenous TAFI mRNA and protein in HepG2 cells. Moreover, the recognition site of miR-143 includes the region containing a commonly occurring single nucleotide polymorphism that is associated with lower plasma TAFI concentrations, providing a plausible mechanistic basis for such effect.

Taken together, our results provide new knowledge about the crucial role of post-transcriptional regulation in mediating TAFI protein levels. Factors that act in *trans* to mediate these effects include both proteins (TTP and HuR) and miRNAs (miR-124, miR-143, miR-506 and miR-708).

To my nephews and niece, Vuk, Maksim and Mila,

for being a constant reminder of life's riches.

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I would like to express my sincere gratitude to my supervisor Dr. Michael B. Boffa for giving me the opportunity to work in his laboratory, and for his support and guidance throughout the past five years. I will always remember Dr. Boffa for being open-minded about new ideas, encouraging my scientific thought and critical thinking. In addition, I would like to thank Dr. Marlys Koschinsky for her guidance throughout my academic career. I would further like to thank Dr. Sirinart Ananvoranich and Dr. Andrew Hubberstey for their guidance as my committee members, as well as all the members of the Boffa and Koschinsky laboratories, past and present, for their helpful suggestions. Especially, I would like to express my sincere gratitude to Roni Hetzel for her assistance in collection of data in Chapter 5. Finally, I would like to thank my family members for their support, with special thanks to my sister, Branna. Thank you all for being there for me.

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List of abbreviations

6-FAM	6-carboxyfluorescein
α2-AP	α2-antiplasmin
APC	activated PC
AUREC	Austrian study on recurrent venous thromboembolism
bp	base pairs
CAD	coronary artery disease
Ch	chapter
CPB2	carboxypeptidase B 2
CPR	carboxypeptidase R
CPU	carboxypeptidase U
DVT	deep vein thrombosis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoresis mobility shift assay
FBS	fetal bovine serum
FDP	fibrin degradation product
fibrin’	partially degraded fibrin
HepG2	human hepatoma cell line
HEX	hexachloro fluorescein
HuR	Human antigen R protein (*ELAV* family member, RNA-binding protein)
IIa	thrombin
IIa/TM	thrombin-thrombomodulin complex
IL-1β	interleukin-1β
IL-6	interleukin-6

IL-8	interleukin-8
IL-10	interleukin-10
IL-13	interleukin 13
kb	kilobase
LETS	leiden thrombophilia study
LPS	Lipopolysaccharide
miR(NA)	microRNA
NFκB	nuclear factor κB
PAI-1	plasminogen activator inhibitor type
PC	protein C
Pg	plasminogen
PLB	passive lysis buffer
Pn	plasmin
polyA	polyadenylation site
PSF	penicillin-streptomycin-Fungizone solution
PTCA	percutaneous transluminal coronary angioplasty
PTCI	potato tuber carboxypeptidase B inhibitor
qRT-PCR	quantitative real-time reverse transcriptase-polymerase chain reaction
RT-PCR	reverse transcriptase-polymerase chain reaction
SNPs	single-nucleotide polymorphisms
TAFI	thrombin-activable fibrinolysis inhibitor
TAFIa	TAFI activated
THP-1	human monocytic leukemia cell line
TM	thrombomodulin
TNFα	tumor necrosis factor

TTP	tristetraprolin
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
UTR	untranslated region
VTE	venous thromboembolism

Chapter 1: General Introduction

The balance between the coagulation and fibrinolytic systems is crucial for normal haemostasis. The respective activities of the two systems are responsible for deposition and removal of fibrin, which is the major structural component of a blood clot. Haemostasis is essential to maintain fluidity of the blood within the vasculature while protecting against excessive blood loss following vascular injury. Thrombin activatable fibrinolysis inhibitor (TAFI) is a plasma zymogen that once activated by thrombin [1], thrombin in complex with thrombomodulin [2] or plasmin [3], regulates the balance between the coagulation and fibrinolytic systems, and also mediates molecular connections between haemostasis and inflammation. TAFI can modulate the inflammatory response through control of pericellular plasminogen activation and modification of inflammatory mediators [4, 5]. Plasma TAFI concentrations vary within the human population, ranging between 50% and 200% of the mean value [6], and the elevated concentrations have been associated with an increased risk for various thrombotic and atherotrombotic disorders, including venous thrombosis [7, 8], acute coronary syndromes [9], myocardial infarction [10] and ischemic stroke [11, 12]. Moreover, several sequence polymorphisms (SNPs) have been identified throughout *CPB2*, the gene encoding TAFI [13, 14]. However, subsequent genetic studies have established that the genetic factors account for only about a quarter of the plasma TAFI variability within the human population [6, 15]. Therefore, the majority of this variation appears to be attributable to transcriptional and post-transcriptional regulatory events that control *CPB2* expression. Moreover, it has been reported that plasma TAFI antigen levels vary as a consequence of age, gender, pregnancy, various disease states and inflammation [16], suggesting a role for hormonal factors and inflammatory mediators in regulating

CPB2 expression. Therefore, the regulation of *CPB2* expression appears to be a crucial mode by which the haemostatic and inflammatory systems respond to the environmental, physiological and pathological challenges.

1.1 Coagulation and Fibrinolysis

The respective activities of deposition and removal of fibrin are mediated by the action of coagulation and fibrinolysis. Upon vascular injury, the first haemostatic structure to form is the platelet plug. Platelets from the circulating blood begin binding to exposed sub-endothelial components of the vessel wall, such as collagen, and a cycle of platelet adhesion, activation, and aggregation ensues, culminating in formation of the platelet plug. The role of the coagulation cascade is to consolidate the clot via formation of fibrin, which is the main structural component of a blood clot. The various reactions of the coagulation cascade are localized to the site of injury by several mechanisms, principally the formation of coagulation cascade complexes on the surface of activated platelets. Proper and precise regulation of coagulation and fibrinolysis cascades, as well as the communication between the cascades is necessary for the development of the haemostatic response at the site of injury, while maintaining the fluidity of the blood throughout the rest of the vasculature. Thrombin (IIa) is the main enzyme product of the coagulation cascade, and is generated by cleavage of prothrombin (Factor II) by the prothrombinase complex. During the initiation phase, small amount of IIa is generated through the extrinsic pathway (tissue-factor dependent pathway), resulting in fibrin formation, but the resultant fibrin is unstable until large amounts of IIa are generated via the intrinsic pathway through activation of the zymogen Factor XI (FXIa) and the cofactors Factor V (FVa) and Factor VIII (FVIIIa) [17]. Once the concentration of IIa reaches 25nM, propagation phase and full activation of the intrinsic pathway ensue,

leading to generation of significant amounts of IIa (reaching up to 850 nM) in a short amount of time [17]. The main substrate of IIa is soluble fibrinogen, which when cleaved by IIa becomes insoluble fibrin. The role of the intrinsic pathway is to generate large amounts of IIa and to stabilize the clot, partly through activation of Factor XIII, which acts as a transglutaminase and introduces covalent crosslinks between fibrin monomers [18], and partly through activation of TAFI [17], which attenuates fibrinolysis and dissolution of the clot.

Another important function of IIa is to activate various anticoagulant mechanisms that stop the deposition of fibrin and attenuate the growth of the clot. The anticoagulant protein C pathway is designed to regulate coagulation, maintain the fluidity of the blood within vasculature, and prevent thrombosis [19-21]. This pathway is activated by proteolytic activation of protein C by IIa at the surface of endothelial cells, and involves two membrane receptors, thrombomodulin (TM) and endothelial protein C receptor (EPCR) [22, 23]. The interaction between IIa and TM shields the procoagulant exosite I of IIa, thus diverting the specificity of IIa from procoagulant substrates (such as fibrinogen) to anticoagulant ones (such as protein C) [24]. Activated protein C (APC) acts as an anticoagulant enzyme by proteolytic inactivation of FVa and FVIIIa, which in turn represents the negative feedback for IIa generation. The APC pathway also confers cytoprotective effects, through binding to EPCR and mediating anti-inflammatory and anti-apoptotic activities, alteration of gene expression and protection of endothelial barrier [25].

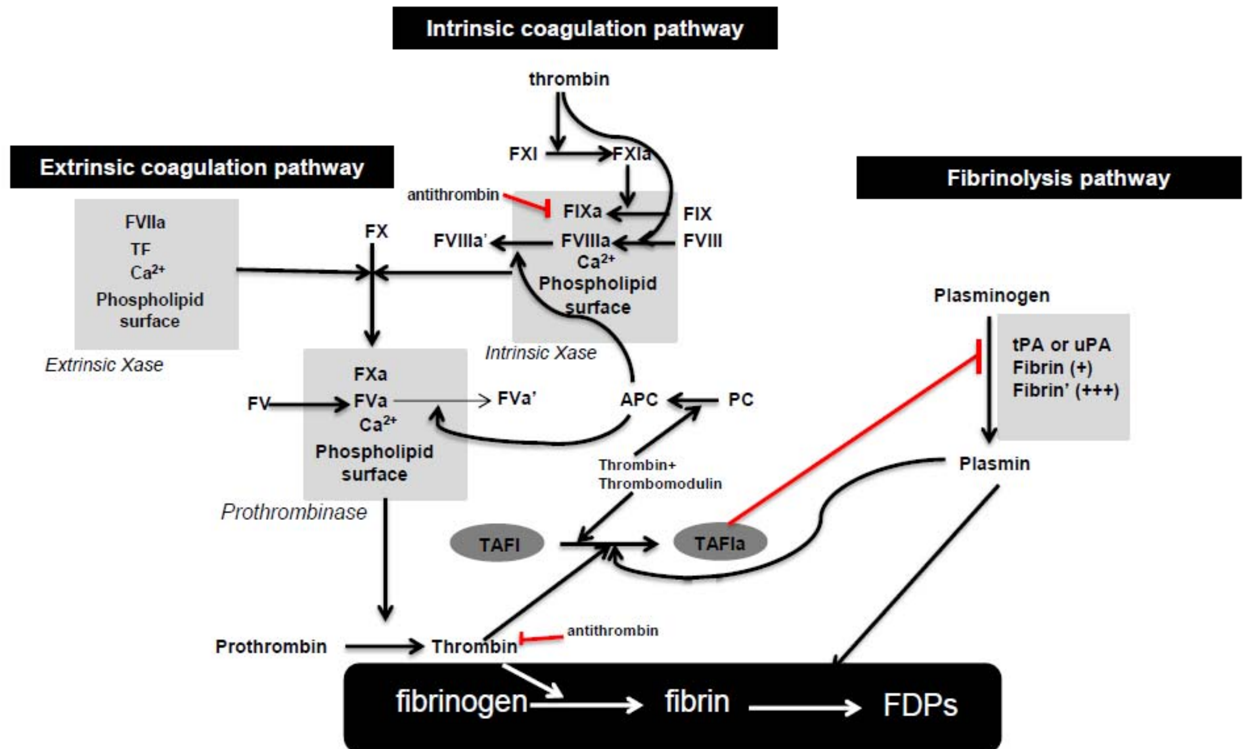

Figure 1.1 TAFI Links the Coagulation and Fibrinolytic Cascades. The role of the extrinsic pathway of coagulation is to generate small amonts of thrombin that promotes fibrin formation, and leads to activation of the intrinsic pathwaym which has evolved to generate large amounts of thrombin and to consolidate the clot, partly through activation of TAFI. The substrate specificitity of thrombin once in complex with thrombomodulin is diverted from fibrinogen and towrds Protein C and TAFI. The dissolution of the clot is executed by the action of plasmin, that is activated from plasminogen by tPA or uPA and fibrin surface plays an essential role in this process. Limited digestion of fibrin by plasmin generates modified fibrin (fibrin') that contains exposed carboxy-terminal lysine residues which make fibrin' a more effective cofactor for plasminogen activation than unmodified fibrin.TAFIa attenuates fibrinolysis by interfering with this positive feedback, by removing carboxy-tereminal lysine residues, thus further modifying fibrin which now exhibits cofactor activity for plasminogen activation that is far lower than even that of inact fibrin. TAFIa thus interferes with plasminogen activation and attenuates fibrinolysis.

Binding of IIa to TM also enhances the substrate specificity of TM towards TAFI, thus also acting as an antifibrinolytic complex. Proteolytic cleavage of TAFI by IIa yields TAFIa, which has basic carboxypeptidase activity that cleaves carboxy-terminal lysine residues from partially degraded fibrin within the clot, acting as a negative regulator of fibrinolysis [26]. This stabilizes the fibrin clot and ensures its localization to the site of injury. The anticoagulant effects of the APC pathway and the antifibrinolytic activity of the TAFI pathway are the two mechanisms that limit the size and growth of the fibrin clot and prevent premature lysis, respectively, and likely precisely regulate the maintenance of the clot. It has been demonstrated that respective concentrations of IIa and TM at different locations dictate the substrate specificity of the complex, and that this specificity is directed towards TAFI at low TM concentrations, and that high TM concentrations promote APC formation, which in turn acts as negative feedback for TAFI activation through inhibition of IIa formation [27].

Dissolution of the clot is effected by the fibrinolytic cascade. Plasmin is the terminal product of the fibrinolytic cascade and is activated through proteolytic cleavage of plasminogen by plasminogen activators such as tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA), although the former can only bind fibrin, which suggests that tPA is the major activator of plasminogen in the fibrinolysis cascade [28]. The ability of tPA to bind to fibrin ensures that fibrinolysis is localized in the vicinity of the fibrin clot where its activity is physiologically required. Fibrin thus acts as a cofactor for conversion of plasminogen to plasmin by tPA. Plasmin degrades fibrin into soluble fibrin degradation products (FDPs) and in the process leaves behind partially degraded fibrin, with exposed carboxyl-terminal lysine residues, which play a central role in a multifaceted positive feedback in the fibrinolysis cascade.

Firstly, the significance of the ability of plasminogen to bind to the carboxy-terminal lysine residues lies in the conversion of plasminogen from a closed from to an opened form, which facilitates its cleavage by tPA [29], generating plasmin. In fact, it has been demonstrated that the modified fibrin serves as a better cofactor for tPA-mediated plasminogen cleavage than the intact counterpart [29-33]. Carboxyl-terminal lysine residues on partially modified fibrin thus mediate positive feedback mechanism in the fibrinolytic cascade. Secondly, modified fibrin also acts as a cofactor for the plasmin-mediated conversion of native Glu-plasminogen to Lys-plasminogen, which is a 20-fold better substrate for activation by tPA [28]. Fibrinolysis can be downregulated by the inhibitors of plasminogen activators or directly by inhibition of plasmin. Plasminogen activator inhibitor type 1 (PAI-1) acts to inhibit tPA, while α2-antiplasmin (α2-AP) exerts its inhibitory effects directly on plasmin. Finally, carboxy-terminal lysine residues reduce the rate of plasmin inhibition by protection of the bound plasmin from inhibition by α2-AP [34, 35].

1.2 The TAFI Pathway as a Link between Coagulation and Fibrinolysis

In keeping with the central role of carboxy-terminal lysine residues in regulation of fibrinolysis, their removal and thus interruption of the positive feedback is also tightly controlled. This control consists of enzymatic cleavage of carboxy-terminal lysine residues by a basic carboxypeptidase, TAFIa. In fact, coagulation and fibrinolytic cascades are interconnected and balanced in such a way that the ultimate product of the coagulation cascade is a pre-requisite for the formation of the ultimate product of the fibrinolytic cascade. Both thrombin and plasmin mediate the links between the two systems [36]. Plasmin proteolytically inactivates FXa [37], thus attenuating the intrinsic pathway of coagulation, and also modifies FVa [38], which can assist in tPA-mediated

plasminogen activation. Thrombin is known to stimulate tPA release by the endothelial cells [39], and it also cleaves TAFI, generating an active form of the enzyme [40]. Activation of TAFI by thrombin is accelerated 1200-fold in the presence of TM [2]. Plasmin can also activate TAFI, albeit with catalytic efficiency well below that of IIa/TM [41], thus making the latter a more likely physiological activator of TAFI. TAFIa cleaves carboxy-terminal lysine residues that are key mediators of the positive feedback in the fibrinolysis cascade, thus protecting the clot and attenuating fibrinolysis. Since TAFI can be activated by thrombin, where thrombin is the end product of the coagulation cascade, and it then acts on partially degraded fibrin to attenuate fibrinolysis, the TAFI pathway represents a direct molecular link between the two cascades.

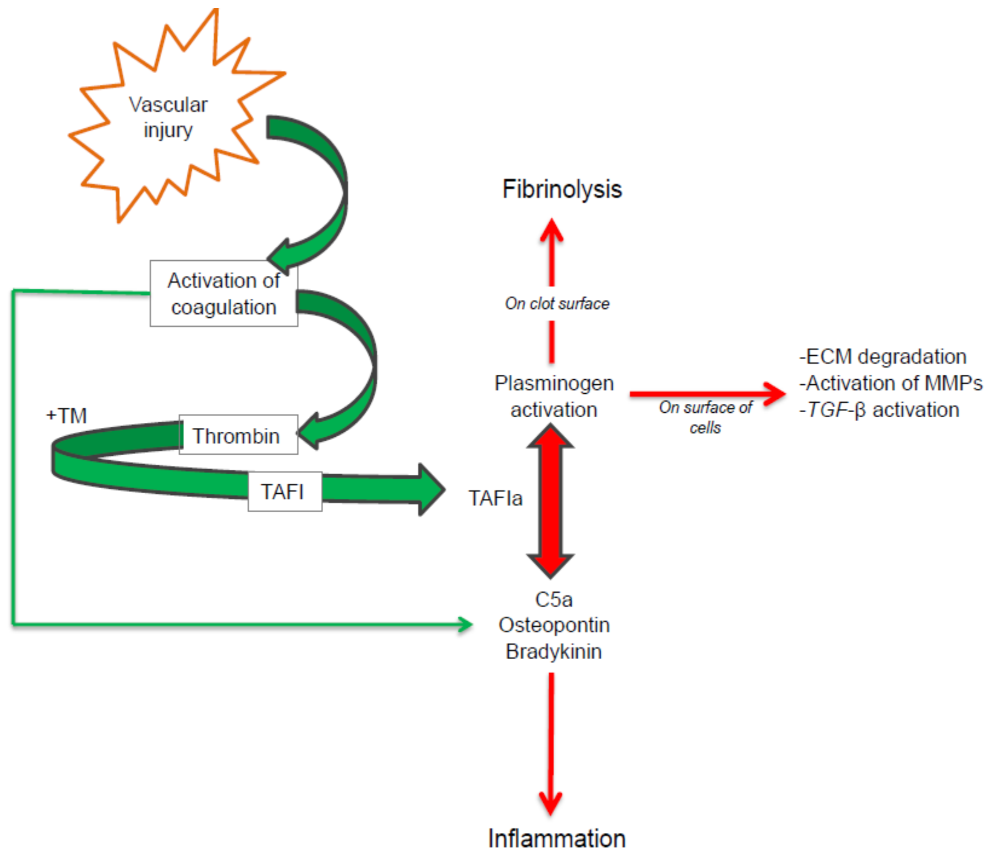

Figure 1.2 TAFIa affects inflammation and plasminogen activation. Activation of TAFI following vascular injury results in alterations in fibrin deposition, pericellular plasminogen activation, deposition of extracellular matrix, and the activity of inflammatory mediators. Inhibition of plasminogen activation on the fibrin surface, through cleavage of carboxy-terminal lysine residues on partially modified fibrin attenuates fibrinolysis. Plasminogen activation is also inhibited on the surface of cells, through cleavage of carboxy-terminal lysine residues on cell surface receptors, which in turn has pleotropic effects in the vasculature. Finally, TAFIa acts as an anti-inflammatory factor via proteolytic inactivation of pro-inflammatory peptides C5a, C3a, bradykinin, and via activation of anti-inflammatory peptide plasmin-cleaved chemerin.

1.3 The TAFI Pathway as a Link between Coagulation and Inflammation

Since protein C and TAFI both act as physiological substrates for the IIa/TM complex, it is not surprising that they also play complementary roles in haemostasis. APC at the site of vascular injury dampens the coagulation cascade, preventing excessive formation of fibrin, while TAFIa serves as an antifibrinolytic factor, stabilizing the clot and preventing premature clot lysis. Additionally, protein C and TAFI also play complementary roles in inflammation. In keeping with the role of thrombin in initiating various pro-inflammatory events, including expression of adhesion molecules, like P-selectin, and activating the NF-κB pathway [42], the regulation of inflammation is achieved by the anti-inflammatory effects of APC and TAFIa. APC is known to inhibit leukocyte adhesion and trafficking, to decrease the synthesis of NF-κB components and cytokine production, and to increase endothelial barrier function [43]. Besides carboxyl-terminal lysine residues on the fibrin surface, additional substrates of TAFIa include various pro-inflammatory mediators, such as bradykinin [44], members of the complement system C3a and C5a [45], thrombin-cleaved osteopontin and plasmin-cleaved chemerin [46].

TAFI has also been shown to remove carboxyl-terminal lysines from cell surface receptors, which would result in attenuation of pericellular plasminogen activation [5]. The proteolytic activity of plasmin is tightly regulated through activation of plasminogen only at specific times and in defined locales, as well as through direct inhibition of plasmin by its natural inhibitors. Plasmin either directly or indirectly, through the activation of certain pro-MMPs, is presumed to hydrolyze many extracellular proteins (the most notable of which is fibrin), proteins of the extracellular matrix and growth factors (such as transforming growth factor β, TGF-β). Generation of plasmin on the cell

surface leads to activation of TGF-β, which in turn results in activation of MMPs and increased cell migration. Attenuation of pericellular plasminogen activation by TAFIa is expected to result in prothrombotic effects in the context of the vascular wall, in keeping with the role of TAFIa as an antifibrinolytic agent. Studies conducted in TAFI knockout mice with hemizygous plasminogen background demonstrated the role of TAFI in modulating pericellular plasminogen activation outside the vascular bed [47]. TAFI deficiency resulted in increased leukocyte migration to the peritoneum following peritoneal thioglycollate injection.

1.4 Properties of TAFI protein

Hendriks and coworkers initially identified an unstable carboxypeptidase in human plasma that was different from the pancreatic carboxypeptidase, and they named it CPU (for "unstable") [48, 49]. Several other groups have thereafter isolated the zymogen form of TAFI, and named it pro-CPR [41] (due to apparent preference for arginine residues in synthetic peptides) or pro-CPB [50] (due to its resemblance to the pancreatic carboxypeptidase B). A few years later, in 1995, Bajzar and coworkers described a human plasma protein capable of attenuating fibrin clot lysis in response to the activation of the intrinsic pathway of coagulation, which they named thrombin activatable fibrinolysis inhibitor, or TAFI [1]. The plasma pool of TAFI is mainly accounted for by the expression of the gene encoding it, *CPB2,* in the liver. TAFI zymogen has an apparent molecular weight of 60 kDa and is glycosylated at four sites on the N-terminal activation peptide (Asn22, Asn51, Asn63, and Asn86) [41, 51]. Upon cleavage of TAFI at Arg92 by IIa [1], IIa in complex with TM [2], or plasmin [3], an active enzyme, TAFIa, is formed, that possesses basic carboxypeptidase activity, with molecular weight of 35 kDa. TAFIa is thermally labile, with half-life of 10 min at body temperature (37^0 C) [51].

Spontaneous conformational change of TAFIa generates an inactive enzyme, TAFIai, that is further cleaved to generate two fragments. There are no known physiological inhibitors of TAFIa, and in fact, intrinsic thermal instability of TAFIa and subsequent cleavage of TAFIai likely represent the physiological mechanisms of its inactivation [52].

Several studies demonstrated that increased TAFI concentrations lead to increased clot lysis time *in vitro* [29, 53, 54], and in fact the kinetic parameters of TAFI activation suggest that the amount of TAFIa generated is directly dependent on TAFI zymogen concentrations. Km for TAFI activation by IIa/TM complex is approximately 1 μM, well above the physiological concentrations of TAFI in the plasma, which are in the nanomolar range [55-57]. Elevated plasma TAFI concentrations might contribute to hypofibrinolytic state, and several clinical studies have subsequently established a link between plasma TAFI levels and cardiovascular disease, as described below.

1.5 Potential Biological Roles of TAFI

Two independent research groups have established that TAFIa attenuates fibrinolysis via a threshold-dependent mechanism [58, 59]. According to this mechanism, fibrinolytic rate remains in its initial phase as long as the concentration of TAFIa remains at or above a key threshold value, and accelerates when TAFIa levels fall below this threshold value [58-61]. Therefore, the factors that influence the time interval over which the concentrations of TAFIa remain above the threshold include the concentration of TAFI zymogen, the rate of its activation by the coagulation cascade, and most importantly by its intrinsic thermal instability. The last factor can be influenced by a naturally occurring variant of TAFI in which Thr325 is replaced by Ile (Thr325Ile), which has a 2-fold longer half-life, and correspondingly greater antifibrinolytic potential [62]. It is likely because of these mechanisms that the extent to which TAFIa can inhibit fibrin

clot lysis is limited, even though it increases with increasing TAFIa concentrations, as demonstrated by several studies.

In vivo studies have also demonstrated the antifibrinolytic potential of TAFIa. Using a rabbit model of tPA-induced thrombolysis, Klement and coworkers demonstrated that inhibition of TAFIa by potato carboxypeptidase inhibitor (PCI) increased clot lysis time, leading to 89% of clot lysis compared to 54% in the absence of PCI [63]. Additionally, the endpoint clot mass decreased only when PCI and t-PA were co-administered, suggesting that the efficacy of thrombolytic therapy may be improved with co-treatment consisting of t-PA and TAFIa inhibitors. Examples of other studies that describe the ability of TAFIa to influence fibrinolysis were conducted in TAFI knockout mice (TAFI$^{-/-}$) [5]. Of note, TAFI $^{-/-}$ mice develop, grow and reproduce normally, and display no overt bleeding tendency [64]. In one study using batroxobin-induced pulmonary embolism model, TAFI $^{-/-}$ mice displayed lower retention of fibrin in the lungs compared to their wild-type counterparts, suggesting enhanced endogenous fibrinolysis in the absence of TAFIa activity [65]. In another study, using arterial thrombolysis model, a specific inhibitor of TAFIa, potato tuber carboxypeptidase inhibitor (PTCI), was shown to accelerate endogenous fibrinolysis [66].

Subsequent studies in TAFI $^{-/-}$ mice have provided valuable insights into the potential biological roles of the TAFI pathway, not only in regulation of fibrinolysis but also in inflammation. These studies have demonstrated that pro-inflammatory mediators bradykinin, the anaphylatoxins, and thrombin-cleaved osteopontin are all substrates for TAFIa *in vivo* [4, 67]. Bradykinin plays a role in regulation of pulmonary and systemic arterial blood pressure, and in vascular permeability. The anaphylatoxins C3a and C5a are members of the complement system that mediate inflammatory response by inducing

release of histamine and cytokines from mast cells. Additionally, they mediate smooth muscle contraction, platelet activation and aggregation [45]. That the anaphylatoxins are a substrate of TAFIa *in vivo* was demonstrated by a study conducted in TAFI $^{-/-}$ mice primed with lipopolysaccharide (LPS); these mice were more susceptible to complement-induced lethality compared to mice expressing TAFI [68]. In an *E.coli*-induced abdominal sepsis mouse model, C5a-mediated neutrophil recruitment into the peritoneum was exaggerated in TAFI $^{-/-}$ mice, while there was no effect on the hemostatic response in these animals [69]. Finally, in an autoimmune arthritis mouse model, injection of anti-collagen antibodies notably increased the severity of arthritis in TAFI $^{-/-}$ animals [70], consistent with the effects of C5a levels.

Indeed, the role of TAFI in regulating inflammation is multifaceted, and consists of both regulation of pro-inflammatory factors described above by TAFIa, as well as the regulation of TAFI expression by various inflammatory mediators. Plasma TAFI levels have been shown to be altered in several inflammatory conditions, including Behcet's disease, inflammatory bowel disease, acute pancreatitis, sepsis and meningococcal disease (reviewed in [16]). Studies in mice have demonstrated that plasma TAFI levels and the abundance of hepatic *CPB2* mRNA were increased during acute phase of inflammation upon injection of LPS into animals [71]. *In vitro* studies conducted in our laboratory have shown that inflammatory mediators modulate *CPB2* expression. Acute phase mediators of inflammation reduced *CPB2* mRNA abundance in HepG2 (human hepatocellular carcinoma) cells, owing to a 2-fold reduction in *CPB2* mRNA half-life, while treatment with glucocorticoids, which exert anti-inflammatory effects, increased *CPB2* mRNA abundance, owing to a 2-fold increase in promoter activity [72].

TAFI $^{-/-}$ mice also display wound healing defects. Excisional cutaneous wounds healed between 89% and 100% in wild-type mice on days 4 and 7 after wounding, respectively. Healing was only accomplished between 38% and 63% in TAFI $^{-/-}$ mice on the same days [73]. This effect is speculated to result from decreased fibrin deposition in cutaneous wounds in the absence of TAFIa activity, which would cause aberrant migration of cells through the matrix, supporting the role of TAFI in regulation of pericellular plasminogen activation. Plasmin generated from activated plasminogen in the context of extracellular matrix mediates fibrin degradation, and fibrin is an essential element of the provisional matrix that allows fibroblasts to migrate into the wound site [74]. Healing of colonic anastomoses in TAFI $^{-/-}$ mice was also impaired. TAFI $^{-/-}$ mice that were subjected to colonic anastomosis surgery displayed decreased strength of the tissue at the site of the suture, and also demonstrated increased weight loss, increased mortality, peritonitis, mesenterial thrombosis and ischemia. The effects of impaired healing of colonic anastomoses are mechanistically less clear than cutaneous wounds, but can likely be explained by defects in plasmin-mediated mechanisms, such as angiogenesis, extracellular matrix remodeling and cell migration, as well as defects in control of inflammation [64]. Another study that supports the role of TAFI in pericellular plasminogen activation investigated the effects of bleomycin-induced lung fibrosis in TAFI $^{-/-}$ mice [75]. Deposition of fibrin in the lungs occurs due to increased proliferation of fibroblasts and collagen-containing cells, as well as excessive accumulation of extracellular matrix in the lung. In keeping with the role of plasmin in mediating degradation of various extracellular matrix components, attenuation of plasminogen activation is expected to have antifibrinolytic consequences. In fact, in this study, TAFI$^{-/-}$

mice were protected from bleomycin-induced lung fibrosis, compared to the heterozygous and wild-type counterparts.

1.6 Association of TAFI with Cardiovascular Disease

Several clinical studies have demonstrated that elevated plasma TAFI concentrations constitute a risk factor for various thrombotic disorders. The Leiden Thrombophilia Study (LETS), a case-control population-based study published two decades ago was the first to demonstrate that elevated TAFI levels are associated with nearly 2-fold increased risk of deep vein thrombosis (DVT) (odds ratio 1.7, $P<0.05$)[76] . It consisted of 474 DVT patients and 474 healthy age- and sex-matched control subjects, and the TAFI plasma levels were measured with electroimmunodiffusion assay using a monoclonal antibody specific for TAFI. In another study, 600 patients were evaluated for a risk of recurrent venous thromboembolism (VTE) with elevated TAFI plasma levels [77]. The patients with first VTE event were selected from the Austrian Study on Recurrent venous Thromboembolism (AUREC), which is an ongoing, prospective, multicenter cohort study designed to evaluate the risk factors for recurrent VTE. This study found that patients with elevated TAFI plasma levels (over 75th percentile in thrombosis patients) are at a 2-fold higher risk for recurrent VTE compared to patients with lover TAFI levels. Moreover, elevated concentrations of TAFI in plasma have been associated with an increased risk of ischemic stroke. A study conducted on 124 patients with a recent ischemic stroke and 125 control subjects matched for age and sex revealed that functional TAFI levels were higher in patients compared to controls (19.5 ± 4.2 vs. 17.7 ± 3.7 min, $P < 0.005$) [78]. Functional TAFI levels were assessed as TAFI-related differences in clot lysis time in the absence or presence of the specific TAFIa inhibitor

(PCI). Patients with TAFI concentrations in the 75th percentile were at a 4-fold higher risk of ischemic stroke ($P < 0.05$) compared to those in the lowest quartile.

While elevated plasma TAFI concentrations are clearly implicated in the development of venous thrombotic events, association of TAFI levels with arterial vascular disease remains obscure. Several clinical studies evaluated the link between TAFI levels and acute coronary artery disease (CAD), and reported contradictory results. One study was conducted at the Coronary Care Unit at the University of Florence, which evaluated 44 consecutively administered patients compared to 44 age- and sex-matched controls and established no difference in plasma TAFI levels in patients compared to controls [79]. On the contrary, another study conducted on 174 patients diagnosed with acute CAD admitted to the Cardiology Unit and 211 healthy controls showed that functional TAFI plasma levels above the 90th percentile are associated with a 4-fold elevated risk of acute CAD [78]. The non-concordant results of these two studies may arise from the different methods used to quantitate TAFI levels, whereby the first study used ELISA detection kit and the second one used an assay that measures TAFI activity, as well as from differences in sample collection and handling. In a large prospective study conducted in France and Northern Ireland, no association between plasma TAFI concentrations and incidence of coronary events was found. Interestingly, a *CPB2* polymorphism associated with higher TAFI concentrations were more frequent in cases in France, but more frequent in controls in Northern Ireland [80].

Subsequently, other studies were conducted to further delineate the role of TAFI in arterial vascular disease. The AtheroGene study was conducted on 1668 individuals with angiographically proven CAD, as part of the prospective AtheroGene cohort [81]. Elevated levels of functional TAFI as measured by the ratio of TAFIa to TAFIai were

associated with increased risk of cardiovascular death (HR = 1.69, P = 0.01). Another study sought to investigate whether Thr325Ile polymorphism and TAFI levels could constitute a risk marker of myocardial infarction (MI) in Egyptian patients [82]. The study included 46 patients with acute MI and 54 age- and sex-matched healthy volunteers. The CT and TT alleles (Thr/Ile and Ile/Ile , respectively) were significantly more frequent in patients compared with controls (54.4% and 32.6% vs. 51.8% and 5.6%, respectively) and were also associated with an increased risk of MI (OR = 4.95; P = 0.0001). Additionally, Ile325 allele was more frequent in patients compared to controls (60.0% vs. 31.5%) (OR = 3.26; P = 0.001). Interestingly, this study also found that TAFI levels were lower in carriers of the TT genotype compared to the CC genotype. No statistically significant relationship was found between Thr325Ile polymorphism and either the type or the site of MI.

A study conducted in the Netherlands was aimed at assessing the role of TAFI in young patients with peripheral arterial disease (PAD) [83]. A total of 47 PAD patients and 141 controls were included and the total plasma TAFI levels were significantly higher in patients (112.4±21.1%) compared to controls (104.9±19.9%, p=0.03). Moreover, the study found that the risk of PAD increased by18% for every 10% increase in TAFI concentration (OR 1.18). Patients with highest functional TAFI levels, above the 90th percentile, were found to be at a 3-fold higher risk for PAD.

The Ludwigshafen Risk and Cardiovascular Health (LURIC) study investigated the influence of TAFI polymorphisms on cardiovascular and thrombotic events [84]. The LURIC study is a prospective cohort study comprising more than 3,300 patients, which demonstrated that the Ile/Ile genotype at position 325 is associated with the incidence of stroke and the age at onset of first stroke in patients with predisposing risk factors for

thrombotic events such as diabetes mellitus, myocardial infarction or hypertension, alone or in combination. In contrast, no significant association was identified for another naturally occurring variant of TAFI, Ala147Thr polymorphism.

A recent study conducted in the Han Chinese population investigated a potential link between atherosclerotic cerebral infarction (ACI) and TAFI levels or SNPs that increase TAFI levels, namely -2345 2G/1G, -1690 A/G, -438 A/G and +1583 A/T [85]. The leading etiological factor for ACI is atherosclerosis. The study was conducted on 225 patients and 184 age-matched healthy controls, and the authors provided preliminary evidence that -2345 2G/1G and -1690 A/G polymorphisms are associated with ACI susceptibility in Han Chinese population. Development of atherosclerotic plaques is also observed as a complication of gouty arthritis (GA). Most recently, a study conducted in Turkey evaluated a potential link between the levels of TAFI and the presence of subclinical atherosclerosis in GA patients [86]. Interestingly, TAFI levels were decreased in GA patients with atherosclerosis compared to GA patients without the complications.

Development and growth of atherosclerotic plaques in the vessel wall is accompanied by secretion of growth factors and cytokines by macrophages and macrophages engorged with surrounding lipid (foam cells) in the plaque. This in turn leads to stimulation of vascular smooth muscle cell growth and synthesis of interstitial collagen [87]. Various pro-atherogenic molecules, such as lipoprotein(a) (Lp(a)), enhance the expression of adhesion molecules (such as ICAM-1) [88]. Additionally, nitric oxide (NO) bioavailability decreases, leading to activation of MMP-2 and MMP-9 [89, 90], and further this reduces inhibition of platelet aggregation [91]. Thus, endothelial dysfunction with reduced NO bioavailability, increased oxidant excess, and expression of adhesion molecules is a major contributor to not only initiation but also to the progression of

atherosclerotic plaque formation and triggering of cardiovascular events. Moreover, activation of plasminogen on the surface of cells is impaired. *In vitro* studies suggest that Lp(a) stimulates the synthesis of PAI-1 by the endothelium, leading to impaired fibrinolysis and pro-thrombotic events [92]. Additionally, reduction of activation of latent transforming growth factor-β (TGF-β) also occurs, possibly mediated by Lp(a), which in turn results in cytokine production and smooth muscle cell proliferation as well as the transformation of these cells into a more atherogenic cellular phenotype [93, 94]. Since the etiology of atherosclerosis involves deregulation of both thrombosis and inflammation, TAFI is a plausible candidate for modulation by or of atherosclerosis.

Two subsequent studies have shown that elevated TAFI levels or *CPB2* polymorphism associated with higher TAFI levels are associated with increased risk of restenosis after percutaneous coronary interventions [95, 96], arguing in favor for the role of TAFI as a regulator of pericellular plasminogen activation and the associated pleotropic effects within the vasculature, such as migration and proliferation of vascular smooth muscle cells and synthesis of extracellular matrix. One study included 159 patients with stable angina who had undergone percutaneous transluminal coronary angioplasty (PTCA) or stenting of the coronary artery and found that pre-procedural TAFI levels were indeed higher in patients with restenosis, up to 2-fold for the patients in the upper tertile, compared to patients in the lower tertile [95]. TAFI levels were in this study measured by commercially available ELISA kit. The second study was a follow-up of the first [96], and its goal was to evaluate the link between the Thr325Ile variant and restenosis rate on the same group of patients. The study found that the T/T allele (Ile/Ile) was associated with lower plasma TAFI levels and lower restenosis rate, despite the increased antifibrinolytic potential of this variant of TAFI. The role of TAFI pathway in

restenosis may therefore be related to the effects on tissue remodeling and cell migration that would be evoked by modulation of pericellular plasminogen activation.

It is clear that additional studies are required to more precisely define the role of TAFI in cardiovascular disease. However, the available data on par suggest a clear pathophysiological relevance for modulation of plasma TAFI concentrations. The exact mechanisms governing these processes also remain obscure, and likely involve the regulation of both fibrinolysis and pericellular plasminogen activation by TAFIa.

1.7 Genotypic and phenotypic variation in TAFI

Plasma TAFI levels vary significantly within the human population, and according to the most recent estimate the extent of the variation ranges between 50% and 200% of the mean value [6]. Subsequent genetic studies have established that only 15-25% of this variability can be attributed to genetic factors [6, 15]. In fact, since the isolation and characterization of *CPB2* in 1999, many SNPs have been identified throughout the gene [13, 14]. Moreover, traditional risk factors for cardiovascular disease have been shown to have insignificant impact on variation in plasma TAFI levels [97]. For example, one study found that age and hypercholesterolemia were both associated with small differences in TAFI levels in women, while no other significant contributors to the variation of TAFI levels were found in both genders [98]. In another study, age was found to be associated with differences in TAFI levels in women but not in men (explained 3% of the variation), and that only in men waist-to-hip ratio was a mild contributor to this variation (explained 2% of the variation) [97]. Therefore, it is tempting to speculate that the remainder of the 75% of the variation in plasma TAFI levels in the human population likely arises from gene regulatory events in *CPB2*. While the identity of these regulatory events remains enigmatic at this point, many disease states and

hormonal factors have been implicated to play a role [16]. These include insulin resistance, glucose intolerance, obesity, thyroid dysfunction, renal disease, gastric cancer, lung cancer, multiple myeloma, various types of inflammatory conditions, such as Behcet's disease, inflammatory bowel disease, and acute pancreatitis. In women, they also extend to include age, pregnancy, pre-eclampsia, use of oral contraceptives and hormone replacement therapy. It is plausible that steroid hormones and inflammatory mediators modulate *CPB2* expression. In fact, our group has recently delineated the mechanistic basis for the post-menopausal rise in plasma TAFI levels and the TAFI-lowering effect of hormone replacement therapy, through suppression of TAFI promoter activity by estrogen and progesterone [99]. To date, however, the mechanisms of regulation of *CPB2* expression by other hormone factors and disease states remain largely unexplored.

1.8 Structure of *CPB2* and Regulation of Expression

The human *CPB2* gene is located on chromosome 13q14.11, and consists of 11 exons spanning approximately 4 kilobases (kb) of genomic DNA [100, 101]. The genes encoding several carboxypeptidases, including rat carboxypeptidases A1, A2, and B and the human mast cell pro-carboxypeptidase A [28], are very similar in exon sizes and the location of the exon-intron boundaries, suggesting that these genes all arose from a common evolutionary ancestor. The +1 position corresponds to the first nucleotide of the cDNA reported by Eaton and coworkers (GenBank accession number AF080222) [41]. The initiator methionine codon is located 18 nucleotides downstream of the +1 nucleotide [100]. Transcription of *CPB2* is initiated at TATA-less promoter, and can begin at several different sites, resulting in 5'-untranslated regions (-UTRs) of different lengths, ranging from 9 to 46 nucleotides [100]. The characterization of *CPB2* gene began in 1999; since

then many new insights into its regulation of expression have been obtained and our group has participated importantly in the process. Initial work from our laboratory mapped the region of the promoter crucial for activity in hepatic cells to about 140 basepairs (bp) upstream of the cluster of transcription start sites, and the key region for basal promoter activity within nucleotides -80 and -73 [100]. Following this report, and using DNaseI footprint analysis, we identified 10 potential transcription factor binding sites, including the ones involved in liver-specific gene transcription: hepatic nuclear factor-1 (HNF-1), nuclear factor Y (NF-Y) and CCAAT/enhancer binding protein (C/EBP). Additionally, we mapped the glucocorticoid responsive element (GRE) between nucleotides -92 and -78 responsible for mediating stimulation of promoter activity by the synthetic glucocorticoid dexamethasone. We also identified the presence of 11 SNPs within the 5'-UTR and found that they have no effect on promoter activity *in vitro*, likely because they are located in the regions that fall between transcription factor binding sites [102]. Therefore, the 5'-UTR SNPs likely do not contribute to the underlying cause of variation in plasma TAFI levels in the human population.

In addition to having 5'-UTR of different lengths, the *CPB2* transcript can also have 3'-UTRs of varying lengths, owing to the existence of 3 potential polyadenylation (polyA) sites. These polyA sites are located at positions +1660, +1693, and +1819, resulting in 3'-UTRs of 390 bp, 423 bp, and 549 bp in length. The relatively long length and the alternative polyadenylation of *CPB2* mRNA argue in favor of the importance of this region in regulation of mRNA stability and abundance. The work conducted in our laboratory previously had contributed a large body of data describing the regulatory role of the 3'-UTR in mediating mRNA stability and the abundance of the transcript [103]. Using a β-globin reporter system, we demonstrated that the 3'-UTR specifically

destabilizes the fusion transcript, and that the coding region plays no role in this process. Additionally, we discovered that the length of the 3'-UTR also plays a role in dictating transcript stability and that longer 3'-UTRs are progressively less stable [103].

We identified three SNPs that occur in the regions common to all 3 polyA forms, +1344 G/A ,+1542 C/G, and +1583 A/T, and we found that they altered the stability of the transcript, contributing to the variation in plasma TAFI concentrations through an effect on hepatic *CPB2* mRNA abundance [13]. Two of the SNPs, +1542 C/G and +1583 A/T have been shown to be associated with plasma TAFI concentrations, while the SNP +1344 G/A has not been studied with respect to association with TAFI antigen concentrations. Nucleotides corresponding to SNPs were introduced either alone or in combination in the context of the β-globin reporter fusion constructs, and based on the pattern of the major haplotypes reported by Henry and coworkers [14]. On balance, the results of this study suggest a causal link between the occurrences of the 3'-UTR SNPs and changes in *CPB2* expression and TAFI protein levels, but are unlikely to be a major contributor to the plasma TAFI concentration variability.

The liver likely represents the major source of TAFI in the plasma, as suggested by reduction in plasma TAFI levels observed in liver diseases [104]. That *CPB2* is expressed at sites other than the liver was initially recognized a decade ago, with a report identifying TAFI protein in human platelets[105]. Using immunofluorescence on permeabilized platelets, Mosnier and colleagues detected a spotted-staining pattern distribution of the TAFI protein within platelets, suggesting its presence in the α granules. They also ruled out the possibility that the platelet TAFI is taken up from the plasma compartment by observing the absence of staining in non-permeabilized platelets. Additional evidence for this comes from the fact that they detected TAFI mRNA in

megakaryocytic cell lines representing the more mature stages of megakaryocytopoiesis, as well as the different glycosylation pattern of platelet-derived TAFI (appeared de-glycosylated, compared to the plasma-derived, glycosylated counterpart). The authors also gained some insights into the properties of the platelet-derived plasma TAFI: its enzymatic activity, activation by thrombin, stimulation of activation by thrombomodulin, inhibition by carboxypeptidase inhibitors, and the thermal instability of TAFIa at 37°C resembled that of TAFI purified from plasma. Recent work from our laboratory has demonstrated that platelet- and plasma-derived TAFI in fact, differ only slightly in molecular mass, certainly to a smaller extent than reported previously [106]. We ascribed this discrepancy to the method of platelet handling and preparation of releasates, mainly due to BSA-mediated effects. It is also possible that subtle differences in the composition of N-linked glycans exist between the two pools of TAFI protein, which may in turn give rise to functional differences. Although the platelet-pool of TAFI comprises only about 0.1% of the plasma pool [105], the dense arrangement and the close contact of platelets at areas of vascular injury could generate appreciable amounts of TAFI within the thrombus microenvironment. Indeed, quantification studies conducted in our laboratory revealed that the intraplatelet concentration of activatable TAFI protein is approximately 40 nM, and increases in TAFIa activity in the nanomolar range have been reported to prolong clot lysis time, platelet-derived TAFI may in fact act as an auxiliary source that could affect thrombolysis. These speculations are in line with the observation of Mutch and colleagues who detected TAFI both along the fibrin fibers and within the platelet-rich areas of thrombi [107], suggesting that the latter might represent platelet-derived TAFI. In keeping with the essential role that platelets play in preventing premature hemostatic plug lysis, such as initiation of clot retraction and secretion of the inhibitors of the

plasminogen system (PAI-1 and α2-antiplasmin), the notion that TAFI may also be released from platelets represents an additional antifibrinolytic strategy upon activation of platelets at regions of vascular damage.

The next line of evidence for extra-hepatic TAFI expression has come from the most recent work in our laboratory. Using RT-PCR, we detected *CPB2* mRNA in the monocytoid cell line THP-1, in macrophages obtained from PMA-induced differentiation of THP-1 monocytes, and in peripheral blood mononuclear cells isolated from human blood; we detected TAFI protein however, only in THP-1 macrophages [108]. The expression of TAFI outside the liver and in cell types directly involved in atherosclerosis, adds another dimension to our understanding of the functions of the TAFI pathway. Since monocytes can differentiate into macrophages within thrombi [109], their contribution of TAFI protein is expected to affect the plasminogen-plasmin system, leading to alterations in both thrombolysis as well as in local cell migration, degradation of the extracellular matrix, activation and secretion of growth factors and cytokines and angiogenesis.

1.9 *CPB2* 3'-UTR as a Crucial Nexus for Regulation of TAFI Expression

Previous work from our laboratory demonstrated that the 3'-UTR of *CPB2* mRNA is capable of mediating regulated changes in mRNA abundance in the presence of pro-inflammatory cytokines [72]. Treatment of HepG2 cells with combination of interleukin-6 (IL-6) and IL-1β resulted in an mRNA abundance-lowering effect. The mechanistic basis for this effect lies in the preferential formation of the longest transcript with the lowest mRNA half-life which is also selectively further destabilized 2-fold in the presence of these pro-inflammatory cytokines. In fact, the TAFI-lowering effect has been observed in experimental endotoxemia patients [110], and may in part be explained by these mechanisms. Other investigators have explored effects of various factors on *CPB2*

mRNA stability. Notably, Ishii and coworkers have demonstrated that forskolin, which increases intracellular cAMP levels, increased *CPB2* mRNA half-life by a factor of 2 [111]. They investigated the possibility that insulin acts as a modulator of cAMP levels that could alter *CPB2* gene expression regulation and found it not to be the case. They have also subsequently demonstrated that WY14643, a PPARα agonist, decreased *CBP2* expression via destabilization of the transcript [112]. This finding is in keeping with the ability of fenofibrate, a hypolipidemic drug and PPARα agonist, to lower TAFI plasma levels in hyperlipidemic patients [113]. Most recently, this group has reported that nobiletin, a polymethoxyflavone found in citrus fruit, reduces TAFI protein secretion by HepG2 cells through effects on promoter activity, without affecting mRNA half-life [114]. They proposed with this study that nobiletin-induced repression of *CPB2* transcription might involve AP-1 inhibition and/or blockage of its binding to the region of the promoter between -119 bp and -99 bp. Therefore, the work of our laboratory and that of others clearly portray *CPB2* 3'-UTR as a crucial nexus in mediating changes in mRNA abundance and thus the amount of TAFI protein produced.

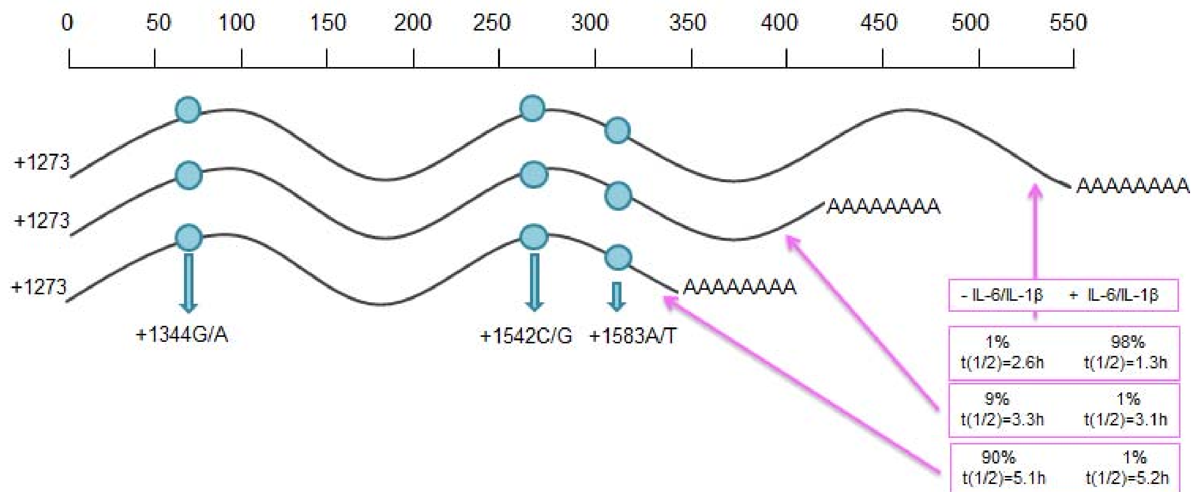

Figure 1.3 Role of the 3'-UTR in modulation of *CPB2* gene expression. Three different polyA sites are used in *CPB2* mRNA processing, leading to 3'-UTR lengths of 549, 393 and 360 nucleotides, respectively. Each polyA site is preceded by a consensus polyA signal sequence. The intrinsic stability of the three differentially polyadenylated forms is different, both under steady-state conditions and in the presence of pro-inflammatory cytokines IL-6 and IL1β. Combination treatment in HepG2 cells results in decrease in *CPB2* mRNA stability and abundance, due to the preferential formation of the longest transcript that is further destabilized by a factor of 2. Shown by blue spheres is the location of the three SNPs in the 3'-UTR. Each of these SNPs influences *CPB2* mRNA stability in a pattern partially consistent with their association with plasma TAFI levels. The 3'-UTR SNPs may directly influence plasma TAFI levels through an effect on *CPB2* mRNA stability.

The importance of the 3'-UTR of transcripts can be appreciated from the fact that they serve as platforms for binding of various cytoplasmic factors that play numerous roles, ranging from protection of degradation, to shuttling among different cellular compartments, and finally to degradation. The latter is effected by a deadenylase complex, CCR4-NOT, which is recruited to the 3'-UTR of target mRNAs by the bound *trans*-acting factors, such as tristetraprolin (TTP) and NANOS2, and mediates deadenylation and subsequent degradation and/or translational repression [115]. The protein factors binding to 3'-UTRs of target mRNAs are referred to as AU-rich element (ARE) binding proteins, and they play regulatory roles in various cellular processes, such as germ cell development in case of NANOS2 [116] and the regulation of inflammatory response, in case of TTP [117]. In addition to the regulatory aspect of controlling the half-life of various transcripts, CCR4-NOT-mediated degradation is also an important part of the cytoplasmic mRNA surveillance system [118]. Nonsense-mediated decay (NMD) is a eukaryotic quality control mechanism that detects aberrant mRNAs containing premature termination codon and induces their rapid degradation. In eukaryotic cells, NMD is mediated by SMG5 and SMG7 proteins, which recruit CCR4-NOT to the target mRNA [119].

In addition to protein factors binding to 3'-UTRs of target transcripts, it has become clear that a class of small non-coding regulatory RNAs, referred to as microRNAs (miRNAs) is capable of targeting mRNAs. The miRNA pathway has been implicated in regulation of many key aspects of gene expression regulation. Most recent evidence suggests that miRNAs are expressed in a tissue-specific manner, that is tightly regulated during embryogenesis and often dysregulated in various pathologies, including cardiovascular diseases [120, 121]. This in turn leads to overexpression or

underexpression of certain miRNAs, consequently affecting abundance of target mRNAs and the protein products they encode. Not surprisingly, most studies are presently aimed at identifying individual miRNAs or miRNA networks involved in pathogenesis of diseases that could potentially serve as biomarkers, and therapeutic strategies to counteract such dysregulation.

MicroRNAs are a class of short, non-coding RNAs that participate in gene expression regulation at the post-transcriptional level. These small, single-stranded polynucleotides, averaging about 20-25 nucleotides in length, are endogenously expressed in animals, plants, and certain viruses, either from the genes encoding them present as clusters or from introns. They regulate physiological cellular processes ranging from proliferation, apoptosis, differentiation, metabolism, and development, as well as pathophysiological processes leading to oncogenesis, cardiovascular diseases and neurodegenerative disorders [122-125].

MiRNA biogenesis can occur either through a canonical or a non-canonical pathway. In the canonical, or classical pathway, miRNAs are transcribed as polycistronic primary transcripts of several hundred nucleotides in length [126] (named pri-miRNAs) by RNA polymerase II [127, 128], although a small group of miRNAs is also transcribed by RNA polymerase III[128, 129]. Pri-miRNAs are processed by a microprocessor enzyme complex consisting of RNAse III enzyme Drosha and its cofactor DiGeorge syndrome critical gene 8 (DGCR8) into precursor miRNAs (pre-miRNAs), approximately 70 nucleotides in length [130]. Once in the pre-miRNA form, they are exported out of the nucleus and into the cytoplasm by exportin 5, and are further cleaved to mature miRNAs 20-25 nucleotides in length, by another RNAse III endonuclease complex, consisting of an enzyme Dicer and its double-stranded RNA binding cofactor TAR RNA binding

protein (TRBP) [131]. Mature miRNA duplexes are separated in the cytoplasm, yielding the guide strand (which becomes the effector miRNA strand) and a passenger strand (miRNA*) that is often degraded [131]. The action of the miRNA strand is effected by formation of the miRNA-induced silencing complex (miRISC) from association of the miRNA strand with RNA-induced silencing complex (RISC) containing argonaute (Ago) proteins [132, 133]. The miRNAs recognize their target mRNAs by complimentary base-pairing with sequences contained most often within the 3'-untranslated region (3'-UTR), and these sequences are designated miRNA recognition elements (MREs) [130]. Depending on the degree of complementarity between miRNAs and MREs, miRNAs repress gene expression by inducing mRNA degradation (in case of perfect base-pairing) or translational repression (in case of imperfect base-pairing). In some cases, MREs are present in the 5'-UTR or the open reading frame, albeit much less frequently than in the 3'-UTR [134]. Degradation of the target mRNA is achieved by deadenylation, followed by 5' to 3' degradation by Xrn1 nuclease. Translational repression can occur by several mechanisms, including deadenylation, blockage of translation elongation, interference by the Ago-RISC complex of binding of translation initiation factors, promotion of premature dissociation of ribosomes and the nascent polypeptide degradation [135, 136].

The non-canonical pathway for miRNA biogenesis is often referred to as the mirtron pathway and includes a group of short introns, called mirtrons, which exist in invertebrates and mammals [137]. The mirtron pathway is microprocessor-independent, and instead involves the splicing machinery. Splicing and debranching of introns generates pre-miRNA hairpins that are suitable substrates for Dicer. In fact, the intron-deriver miRNAs have been identified in fish, chicken embryos and mice, in addition to

mammalian cells, suggesting evolutionary conservation of this mechanism for gene regulation *in vivo* [138, 139].

Abundance of specific miRNA is dictated by the rates of their transcription, processing and decay. Transcription rate is dependent on the methylation status of their promoters, in similar manner to many protein coding genes [131, 140]. Processing of miRNAs can be affected at multiple steps during biogenesis, including availability of Ago proteins, uridylation of the miRNA 3' ends, which could enhance or inhibit Drosha-DGCR8 and Dicer-TRBP processing steps, while miRNA editing can change complementarity to target mRNA sequences [141-143]. Since the mechanisms that control the miRNA turnover are not yet fully understood, other modifications that stabilize or destabilize miRNAs may also exist. Interestingly, miRNAs are unusually stable in plasma and are resistant to many harsh conditions, such as boiling, low and high pH, repeated freezing and thawing cycles and long storage [144-147]. They have been found to exist in various body fluids, such as tears, saliva, urine and breast milk. The stability of miRNAs in the circulation can be attributed to their association with lipid-based carriers, either associated or nonassociated to vesicles. The latter accounts for 80-90% of circulating miRNAs, that associate with RNA-binding proteins such as Ago2 and nuclephosmine 1(NPM1) in very stable complexes, or with lipoprotein complexes, such as high-density lipoprotein (HDL) [146-148]. The vesicular forms of miRNAs are incorporated into exosomes and microvesicles derived from multivesicular bodies, which are then released from the cell and participate in cell-cell communication and transfer of genetic material [146, 147, 149]. As such, they participate in regulation of immunity, angiogenesis and cell migration. Aberrant regulation of miRNA processing and trafficking can have deleterious consequences in normal cellular function.

1.10 Rationale, Hypothesis and Objectives

Significant variation in plasma TAFI concentration exists in the human population and genetic factors explain only 15-25% of this variability. The remaining 75% of the variation likely arises from differences in gene regulatory events, such as those mediated at the levels of post-transcriptional regulation. The relatively long length and the presence of three alternative polyadenylation sites within the *CPB2* 3'-UTR suggests the role of this region in regulation of *CPB2* mRNA abundance through modulation of mRNA stability and/or polyadenylation pattern. The identity of factors that act in *trans*, and the *cis*-sequences they recognize are still enigmatic. Regulation of *CPB2* expression might have considerable implications for haemostasis, inflammation, and other processes in both health and disease. Additionally, new knowledge describing extra-hepatic TAFI expression suggests that the TAFI pathway may be involved in processes beyond clot lysis, such as those that take place in the vessel wall in atherosclerosis. Understanding the molecular mechanisms underlying regulation of *CPB2* expression, both in hepatic and extra-hepatic tissues, will aid in deciphering results of numerous clinical studies that reported modulation of plasma TAFI levels in various disease states and pathologies. For these reasons, analysis of the mechanisms underlying regulation of tissue-specific *CPB2* expression, both constitutive and regulated, represents a crucial piece of a puzzle revealing the full spectrum of TAFI pathway functions.

Therefore, the specific hypotheses to be tested are: 1) *CPB2* 3'-UTR harbours (in)stability determinants in form of *cis*-acting sequences which are recognized by specific *trans*-acting factors, 2) *trans*-acting regulatory factors exert their effects post-transcriptionally, affecting mRNA abundance through modulation of mRNA stability and polyadenylation site selection, and 3) in addition to protein factors acting on *CPB2*

mRNA in *trans*, small regulatory RNA molecules, such as microRNAs, also mediate constitutive and regulated post-transcriptional regulation.

The first main objective of this thesis is aimed at characterization of *CPB2* 3'-UTR with respect to regulatory sequences that mediate binding of *trans*-acting protein factors that exert stabilizing and/or destabilizing effects. The second main objective is centered on elucidating the post-transcriptional mechanisms mediated by various *trans*-acting factors in modulating mRNA abundance and thus TAFI protein levels in both HepG2 cells and THP-1 macrophages in the presence of various inflammatory stimuli. The third main objective is to investigate the regulatory mechanisms of microRNA pathway in constitutive and regulated *CPB2* gene expression regulation.

1.11 References

1. Bajzar, L., R. Manuel, and M.E. Nesheim, *Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor.* J Biol Chem, 1995. **270**(24): p. 14477-84.
2. Bajzar, L., J. Morser, and M. Nesheim, *TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex.* J Biol Chem, 1996. **271**(28): p. 16603-8.
3. Mao, S.S., et al., *Characterization of plasmin-mediated activation of plasma procarboxypeptidase B. Modulation by glycosaminoglycans.* J Biol Chem, 1999. **274**(49): p. 35046-52.
4. Leung, L.L., et al., *Regulation of tissue inflammation by thrombin-activatable carboxypeptidase B (or TAFI).* Mol Immunol, 2008. **45**(16): p. 4080-3.
5. Morser, J., et al., *What has been learnt from the thrombin-activatable fibrinolysis inhibitor-deficient mouse?* J Thromb Haemost, 2010. **8**(5): p. 868-76.
6. Frere, C., et al., *Quantification of thrombin activatable fibrinolysis inhibitor (TAFI) gene polymorphism effects on plasma levels of TAFI measured with assays insensitive to isoform-dependent artefact.* Thromb Haemost, 2005. **94**(2): p. 373-9.
7. van Tilburg, N.H., F.R. Rosendaal, and R.M. Bertina, *Thrombin activatable fibrinolysis inhibitor and the risk for deep vein thrombosis.* Blood, 2000. **95**(9): p. 2855-9.
8. Meltzer, M.E., et al., *Venous thrombosis risk associated with plasma hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1.* Blood, 2010. **116**(1): p. 113-21.
9. Schroeder, V., et al., *Thrombin activatable fibrinolysis inhibitor (TAFI) levels in patients with coronary artery disease investigated by angiography.* Thromb Haemost, 2002. **88**(6): p. 1020-5.
10. Zorio, E., et al., *Thrombin-activatable fibrinolysis inhibitor in young patients with myocardial infarction and its relationship with the fibrinolytic function and the protein C system.* Br J Haematol, 2003. **122**(6): p. 958-65.
11. Leebeek, F.W., et al., *High functional levels of thrombin-activatable fibrinolysis inhibitor are associated with an increased risk of first ischemic stroke.* J Thromb Haemost, 2005. **3**(10): p. 2211-8.
12. Ladenvall, C., et al., *Thrombin activatable fibrinolysis inhibitor activation peptide shows association with all major subtypes of ischemic stroke and with TAFI gene variation.* Arterioscler Thromb Vasc Biol, 2007. **27**(4): p. 955-62.
13. Boffa, M.B., et al., *Effect of single nucleotide polymorphisms on expression of the gene encoding thrombin-activatable fibrinolysis inhibitor: a functional analysis.* Blood, 2008. **111**(1): p. 183-9.
14. Henry, M., et al., *Identification of polymorphisms in the promoter and the 3' region of the TAFI gene: evidence that plasma TAFI antigen levels are strongly genetically controlled.* Blood, 2001. **97**(7): p. 2053-8.

15. Frere, C., et al., *Fine mapping of quantitative trait nucleotides underlying thrombin-activatable fibrinolysis inhibitor antigen levels by a transethnic study.* Blood, 2006. **108**(5): p. 1562-8.
16. Boffa, M.B. and M.L. Koschinsky, *Curiouser and curiouser: recent advances in measurement of thrombin-activatable fibrinolysis inhibitor (TAFI) and in understanding its molecular genetics, gene regulation, and biological roles.* Clin Biochem, 2007. **40**(7): p. 431-42.
17. Nesheim, M., *Thrombin and fibrinolysis.* Chest, 2003. **124**(3 Suppl): p. 33S-9S.
18. Lorand, L., *Crosslinks in blood: transglutaminase and beyond.* FASEB J, 2007. **21**(8): p. 1627-32.
19. Dahlback, B. and B.O. Villoutreix, *Regulation of blood coagulation by the protein C anticoagulant pathway: novel insights into structure-function relationships and molecular recognition.* Arterioscler Thromb Vasc Biol, 2005. **25**(7): p. 1311-20.
20. Weiler, H., *Regulation of inflammation by the protein C system.* Crit Care Med, 2010. **38**(2 Suppl): p. S18-25.
21. Rezaie, A.R., *Regulation of the protein C anticoagulant and antiinflammatory pathways.* Curr Med Chem, 2010. **17**(19): p. 2059-69.
22. Fukudome, K. and C.T. Esmon, *Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor.* J Biol Chem, 1994. **269**(42): p. 26486-91.
23. Esmon, C.T. and W.G. Owen, *The discovery of thrombomodulin.* J Thromb Haemost, 2004. **2**(2): p. 209-13.
24. Stearns-Kurosawa, D.J., et al., *The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex.* Proc Natl Acad Sci U S A, 1996. **93**(19): p. 10212-6.
25. Mosnier, L.O., B.V. Zlokovic, and J.H. Griffin, *The cytoprotective protein C pathway.* Blood, 2007. **109**(8): p. 3161-72.
26. Cote, H.C., et al., *Functional characterization of recombinant human meizothrombin and Meizothrombin(desF1). Thrombomodulin-dependent activation of protein C and thrombin-activatable fibrinolysis inhibitor (TAFI), platelet aggregation, antithrombin-III inhibition.* J Biol Chem, 1997. **272**(10): p. 6194-200.
27. Mosnier, L.O., J.C. Meijers, and B.N. Bouma, *Regulation of fibrinolysis in plasma by TAFI and protein C is dependent on the concentration of thrombomodulin.* Thromb Haemost, 2001. **85**(1): p. 5-11.
28. Hoylaerts, M., et al., *Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin.* J Biol Chem, 1982. **257**(6): p. 2912-9.
29. Wang, W., et al., *A study of the mechanism of inhibition of fibrinolysis by activated thrombin-activable fibrinolysis inhibitor.* J Biol Chem, 1998. **273**(42): p. 27176-81.
30. Sakharov, D.V., E.F. Plow, and D.C. Rijken, *On the mechanism of the antifibrinolytic activity of plasma carboxypeptidase B.* J Biol Chem, 1997. **272**(22): p. 14477-82.
31. Medved, L. and W. Nieuwenhuizen, *Molecular mechanisms of initiation of fibrinolysis by fibrin.* Thromb Haemost, 2003. **89**(3): p. 409-19.

32. Fleury, V. and E. Angles-Cano, *Characterization of the binding of plasminogen to fibrin surfaces: the role of carboxy-terminal lysines.* Biochemistry, 1991. **30**(30): p. 7630-8.
33. Pannell, R., J. Black, and V. Gurewich, *Complementary modes of action of tissue-type plasminogen activator and pro-urokinase by which their synergistic effect on clot lysis may be explained.* J Clin Invest, 1988. **81**(3): p. 853-9.
34. Schneider, M., et al., *Activated thrombin-activatable fibrinolysis inhibitor reduces the ability of high molecular weight fibrin degradation products to protect plasmin from antiplasmin.* J Biol Chem, 2004. **279**(14): p. 13340-5.
35. Schneider, M. and M. Nesheim, *A study of the protection of plasmin from antiplasmin inhibition within an intact fibrin clot during the course of clot lysis.* J Biol Chem, 2004. **279**(14): p. 13333-9.
36. Boffa, M.B., M.E. Nesheim, and M.L. Koschinsky, *Thrombin activable fibrinolysis inhibitor (TAFI): molecular genetics of an emerging potential risk factor for thrombotic disorders.* Curr Drug Targets Cardiovasc Haematol Disord, 2001. **1**(2): p. 59-74.
37. Pryzdial, E.L., et al., *Plasmin converts factor X from coagulation zymogen to fibrinolysis cofactor.* J Biol Chem, 1999. **274**(13): p. 8500-5.
38. Pryzdial, E.L., L. Bajzar, and M.E. Nesheim, *Prothrombinase components can accelerate tissue plasminogen activator-catalyzed plasminogen activation.* J Biol Chem, 1995. **270**(30): p. 17871-7.
39. Fukushima, M., Y. Nakashima, and K. Sueishi, *Thrombin enhances release of tissue plasminogen activator from bovine corneal endothelial cells.* Invest Ophthalmol Vis Sci, 1989. **30**(7): p. 1576-83.
40. Bajzar, L. and M. Nesheim, *The effect of activated protein C on fibrinolysis in cell-free plasma can be attributed specifically to attenuation of prothrombin activation.* J Biol Chem, 1993. **268**(12): p. 8608-16.
41. Eaton, D.L., et al., *Isolation, molecular cloning, and partial characterization of a novel carboxypeptidase B from human plasma.* J Biol Chem, 1991. **266**(32): p. 21833-8.
42. Coughlin, S.R., *Thrombin signalling and protease-activated receptors.* Nature, 2000. **407**(6801): p. 258-64.
43. Esmon, C.T., *Protein C anticoagulant system--anti-inflammatory effects.* Semin Immunopathol, 2012. **34**(1): p. 127-32.
44. Hendriks, D., et al., *Human serum carboxypeptidase U: a new kininase?* Agents Actions Suppl, 1992. **38 (Pt 1)**: p. 407-13.
45. Campbell, W.D., et al., *Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N.* Microbiol Immunol, 2002. **46**(2): p. 131-4.
46. Du, X.Y., et al., *Regulation of chemerin bioactivity by plasma carboxypeptidase N, carboxypeptidase B (activated thrombin-activable fibrinolysis inhibitor), and platelets.* J Biol Chem, 2009. **284**(2): p. 751-8.
47. Swaisgood, C.M., et al., *In vivo regulation of plasminogen function by plasma carboxypeptidase B.* J Clin Invest, 2002. **110**(9): p. 1275-82.
48. Hendriks, D., et al., *Characterisation of a carboxypeptidase in human serum distinct from carboxypeptidase N.* J Clin Chem Clin Biochem, 1989. **27**(5): p. 277-85.

49. Hendriks, D., et al., *A labile enzyme in fresh human serum interferes with the assay of carboxypeptidase N.* Clin Chem, 1989. **35**(1): p. 177.
50. Campbell, W. and H. Okada, *An arginine specific carboxypeptidase generated in blood during coagulation or inflammation which is unrelated to carboxypeptidase N or its subunits.* Biochem Biophys Res Commun, 1989. **162**(3): p. 933-9.
51. Boffa, M.B., et al., *Plasma and recombinant thrombin-activable fibrinolysis inhibitor (TAFI) and activated TAFI compared with respect to glycosylation, thrombin/thrombomodulin-dependent activation, thermal stability, and enzymatic properties.* J Biol Chem, 1998. **273**(4): p. 2127-35.
52. Boffa, M.B., et al., *Roles of thermal instability and proteolytic cleavage in regulation of activated thrombin-activable fibrinolysis inhibitor.* J Biol Chem, 2000. **275**(17): p. 12868-78.
53. Broze, G.J., Jr. and D.A. Higuchi, *Coagulation-dependent inhibition of fibrinolysis: role of carboxypeptidase-U and the premature lysis of clots from hemophilic plasma.* Blood, 1996. **88**(10): p. 3815-23.
54. Von dem Borne, P.A., et al., *Thrombin-mediated activation of factor XI results in a thrombin-activatable fibrinolysis inhibitor-dependent inhibition of fibrinolysis.* J Clin Invest, 1997. **99**(10): p. 2323-7.
55. Guo, X., et al., *Arginine carboxypeptidase (CPR) in human plasma determined with sandwich ELISA.* Microbiol Immunol, 1999. **43**(7): p. 691-8.
56. Bajzar, L., M.E. Nesheim, and P.B. Tracy, *The profibrinolytic effect of activated protein C in clots formed from plasma is TAFI-dependent.* Blood, 1996. **88**(6): p. 2093-100.
57. Chetaille, P., et al., *Plasma TAFI antigen variations in healthy subjects.* Thromb Haemost, 2000. **83**(6): p. 902-5.
58. Leurs, J., et al., *Carboxypeptidase U (TAFIa) prevents lysis from proceeding into the propagation phase through a threshold-dependent mechanism.* J Thromb Haemost, 2004. **2**(3): p. 416-23.
59. Walker, J.B. and L. Bajzar, *The intrinsic threshold of the fibrinolytic system is modulated by basic carboxypeptidases, but the magnitude of the antifibrinolytic effect of activated thrombin-activable fibrinolysis inhibitor is masked by its instability.* J Biol Chem, 2004. **279**(27): p. 27896-904.
60. Knecht, W., et al., *Limited mutagenesis increases the stability of human carboxypeptidase U (TAFIa) and demonstrates the importance of CPU stability over proCPU concentration in down-regulating fibrinolysis.* FEBS J, 2006. **273**(4): p. 778-92.
61. Walker, J.B. and L. Bajzar, *Complete inhibition of fibrinolysis by sustained carboxypeptidase B activity: the role and requirement of plasmin inhibitors.* J Thromb Haemost, 2007. **5**(6): p. 1257-64.
62. Schneider, M., et al., *Two naturally occurring variants of TAFI (Thr-325 and Ile-325) differ substantially with respect to thermal stability and antifibrinolytic activity of the enzyme.* J Biol Chem, 2002. **277**(2): p. 1021-30.
63. Klement, P., P. Liao, and L. Bajzar, *A novel approach to arterial thrombolysis.* Blood, 1999. **94**(8): p. 2735-43.
64. Nagashima, M., et al., *Thrombin-activatable fibrinolysis inhibitor (TAFI) deficiency is compatible with murine life.* J Clin Invest, 2002. **109**(1): p. 101-10.

65. Mao, S.S., et al., *Demonstration of enhanced endogenous fibrinolysis in thrombin activatable fibrinolysis inhibitor-deficient mice.* Blood Coagul Fibrinolysis, 2005. **16**(6): p. 407-15.
66. Hashimoto, M., et al., *Enhancement of endogenous plasminogen activator-induced thrombolysis by argatroban and APC and its control by TAFI, measured in an arterial thrombolysis model in vivo using rat mesenteric arterioles.* Thromb Haemost, 2002. **87**(1): p. 110-3.
67. Myles, T., et al., *Thrombin activatable fibrinolysis inhibitor, a potential regulator of vascular inflammation.* J Biol Chem, 2003. **278**(51): p. 51059-67.
68. Asai, S., et al., *Absence of procarboxypeptidase R induces complement-mediated lethal inflammation in lipopolysaccharide-primed mice.* J Immunol, 2004. **173**(7): p. 4669-74.
69. Renckens, R., et al., *Absence of thrombin-activatable fibrinolysis inhibitor protects against sepsis-induced liver injury in mice.* J Immunol, 2005. **175**(10): p. 6764-71.
70. Song, J.J., et al., *Plasma carboxypeptidase B downregulates inflammatory responses in autoimmune arthritis.* J Clin Invest, 2011. **121**(9): p. 3517-27.
71. Sato, T., et al., *Pro-carboxypeptidase R is an acute phase protein in the mouse, whereas carboxypeptidase N is not.* J Immunol, 2000. **165**(2): p. 1053-8.
72. Boffa, M.B., et al., *Acute phase mediators modulate thrombin-activable fibrinolysis inhibitor (TAFI) gene expression in HepG2 cells.* J Biol Chem, 2003. **278**(11): p. 9250-7.
73. te Velde, E.A., et al., *Impaired healing of cutaneous wounds and colonic anastomoses in mice lacking thrombin-activatable fibrinolysis inhibitor.* J Thromb Haemost, 2003. **1**(10): p. 2087-96.
74. Boffa, M.B., *TAFI and wound healing: closing a knowledge gap.* J Thromb Haemost, 2003. **1**(10): p. 2075-7.
75. Fujimoto, H., et al., *Thrombin-activatable fibrinolysis inhibitor deficiency attenuates bleomycin-induced lung fibrosis.* Am J Pathol, 2006. **168**(4): p. 1086-96.
76. Koster, T., et al., *Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study.* Lancet, 1993. **342**(8886-8887): p. 1503-6.
77. Eichinger, S., et al., *Thrombin-activatable fibrinolysis inhibitor and the risk for recurrent venous thromboembolism.* Blood, 2004. **103**(10): p. 3773-6.
78. Santamaria, A., et al., *Risk of acute coronary artery disease associated with functional thrombin activatable fibrinolysis inhibitor plasma level.* Haematologica, 2004. **89**(7): p. 880-1.
79. Paola Cellai, A., et al., *TAFI activity and antigen plasma levels are not increased in acute coronary artery disease patients admitted to a coronary care unit.* Thromb Res, 2006. **118**(4): p. 495-500.
80. Morange, P.E., et al., *TAFI gene haplotypes, TAFI plasma levels and future risk of coronary heart disease: the PRIME Study.* J Thromb Haemost, 2005. **3**(7): p. 1503-10.
81. Tregouet, D.A., et al., *Activated thrombin activatable fibrinolysis inhibitor levels are associated with the risk of cardiovascular death in patients with coronary artery disease: the AtheroGene study.* J Thromb Haemost, 2009. **7**(1): p. 49-57.

82. Kamal, H.M., et al., *Plasma thrombin-activatable fibrinolysis inhibitor levels and Thr325Ile polymorphism as a risk marker of myocardial infarction in Egyptian patients.* Acta Cardiol, 2011. **66**(4): p. 483-8.
83. de Bruijne, E.L., et al., *High thrombin activatable fibrinolysis inhibitor levels are associated with an increased risk of premature peripheral arterial disease.* Thromb Res, 2011. **127**(3): p. 254-8.
84. Kozian, D.H., et al., *Association between the Thr325Ile polymorphism of the thrombin-activatable fibrinolysis inhibitor and stroke in the Ludwigshafen Risk and Cardiovascular Health Study.* Thromb Haemost, 2010. **103**(5): p. 976-83.
85. Li, Y., et al., *Association between polymorphisms in the flanking region of the TAFI gene and atherosclerotic cerebral infarction in a Chinese population.* Lipids Health Dis, 2014. **13**: p. 80.
86. Uyanik, M.S., et al., *Tissue factor pathway inhibitor and thrombin-activatable carboxypeptidase B for prediction of early atherosclerosis in gouty arthritis.* Thromb Res, 2014.
87. Libby, P., *Inflammation in atherosclerosis.* Nature, 2002. **420**(6917): p. 868-74.
88. Lawn, R.M., et al., *Atherogenesis in transgenic mice expressing human apolipoprotein(a).* Nature, 1992. **360**(6405): p. 670-2.
89. Uemura, S., et al., *Diabetes mellitus enhances vascular matrix metalloproteinase activity: role of oxidative stress.* Circ Res, 2001. **88**(12): p. 1291-8.
90. Eberhardt, W., et al., *Nitric oxide modulates expression of matrix metalloproteinase-9 in rat mesangial cells.* Kidney Int, 2000. **57**(1): p. 59-69.
91. Radomski, M.W., R.M. Palmer, and S. Moncada, *Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium.* Lancet, 1987. **2**(8567): p. 1057-8.
92. Aznar, J., et al., *Euglobulin clot lysis induced by tissue type plasminogen activator in subjects with increased levels and different isoforms of lipoprotein (a).* Thromb Res, 1993. **72**(5): p. 459-65.
93. Smith, E.B., G.A. Keen, and A. Grant, *Factors influencing the accumulation in fibrous plaques of lipid derived from low density lipoprotein. I. Relation between fibrin and immobilization of apo B-containing lipoprotein.* Atherosclerosis, 1990. **84**(2-3): p. 165-71.
94. Beisiegel, U., et al., *Lipoprotein(a) in the arterial wall.* Eur Heart J, 1990. **11 Suppl E**: p. 174-83.
95. Lau, H.K., et al., *Thrombin-activatable fibrinolysis inhibitor (TAFI): a novel predictor of angiographic coronary restenosis.* Thromb Haemost, 2003. **90**(6): p. 1187-91.
96. Segev, A., et al., *Thr325Ile polymorphism of the TAFI gene is related to TAFI antigen plasma levels and angiographic restenosis after percutaneous coronary interventions.* Thromb Res, 2004. **114**(2): p. 137-41.
97. Juhan-Vague, I., et al., *Thrombin-activatable fibrinolysis inhibitor antigen levels and cardiovascular risk factors.* Arterioscler Thromb Vasc Biol, 2000. **20**(9): p. 2156-61.
98. Santamaria, A., et al., *Association of functional thrombin-activatable fibrinolysis inhibitor (TAFI) with conventional cardiovascular risk factors and its correlation with other hemostatic factors in a Spanish population.* Am J Hematol, 2004. **76**(4): p. 348-52.

99. Garand, M., et al., *Regulation of the gene encoding human thrombin-activatable fibrinolysis inhibitor by estrogen and progesterone.* Blood Coagul Fibrinolysis, 2013. **24**(4): p. 393-404.
100. Boffa, M.B., et al., *Characterization of the gene encoding human TAFI (thrombin-activable fibrinolysis inhibitor; plasma procarboxypeptidase B).* Biochemistry, 1999. **38**(20): p. 6547-58.
101. Vanhoof, G., et al., *The gene for human carboxypeptidase U (CPU)--a proposed novel regulator of plasminogen activation--maps to 13q14.11.* Genomics, 1996. **38**(3): p. 454-5.
102. Garand, M., et al., *Molecular analysis of the human thrombin-activatable fibrinolysis inhibitor gene promoter.* Br J Haematol, 2007. **138**(2): p. 231-44.
103. Maret, D., et al., *Role of mRNA transcript stability in modulation of expression of the gene encoding thrombin activable fibrinolysis inhibitor.* J Thromb Haemost, 2004. **2**(11): p. 1969-79.
104. Van Thiel, D.H., M. George, and J. Fareed, *Low levels of thrombin activatable fibrinolysis inhibitor (TAFI) in patients with chronic liver disease.* Thromb Haemost, 2001. **85**(4): p. 667-70.
105. Mosnier, L.O., et al., *Identification of thrombin activatable fibrinolysis inhibitor (TAFI) in human platelets.* Blood, 2003. **101**(12): p. 4844-6.
106. Schadinger, S.L., et al., *Secretion and antifibrinolytic function of thrombin-activatable fibrinolysis inhibitor from human platelets.* J Thromb Haemost, 2010. **8**(11): p. 2523-9.
107. Mutch, N.J., et al., *Thrombus lysis by uPA, scuPA and tPA is regulated by plasma TAFI.* J Thromb Haemost, 2003. **1**(9): p. 2000-7.
108. Lin, J.H., et al., *Identification of human thrombin-activatable fibrinolysis inhibitor in vascular and inflammatory cells.* Thromb Haemost, 2011. **105**(6): p. 999-1009.
109. Leu, H.J., et al., *Differentiation of mononuclear blood cells into macrophages, fibroblasts and endothelial cells in thrombus organization.* Exp Cell Biol, 1988. **56**(4): p. 201-10.
110. Verbon, A., et al., *Effects of IC14, an anti-CD14 antibody, on coagulation and fibrinolysis during low-grade endotoxemia in humans.* J Infect Dis, 2003. **187**(1): p. 55-61.
111. Ishii, H., et al., *Expression of thrombin-activatable fibrinolysis inhibitor (TAFI) is up-regulated by increase in intracellular cyclic AMP levels in cultured HepG2 cells.* Thromb Haemost, 2009. **102**(6): p. 1204-11.
112. Masuda, Y., et al., *Peroxisome proliferator-activated receptor-alpha agonists repress expression of thrombin-activatable fibrinolysis inhibitor by decreasing transcript stability.* Thromb Haemost, 2012. **108**(1): p. 74-85.
113. Kilicarslan, A., et al., *Fenofibrate improves endothelial function and decreases thrombin-activatable fibrinolysis inhibitor concentration in metabolic syndrome.* Blood Coagul Fibrinolysis, 2008. **19**(4): p. 310-4.
114. Takada, K., et al., *Nobiletin, a polymethoxyflavone in citrus fruits, reduces TAFI expression in HepG2 cells through transcriptional inhibition.* Thromb Haemost, 2013. **109**(6): p. 1060-9.
115. Wahle, E. and G.S. Winkler, *RNA decay machines: deadenylation by the Ccr4-not and Pan2-Pan3 complexes.* Biochim Biophys Acta, 2013. **1829**(6-7): p. 561-70.

116. Tsuda, M., et al., *Conserved role of nanos proteins in germ cell development.* Science, 2003. **301**(5637): p. 1239-41.
117. Tiedje, C., et al., *The p38/MK2-driven exchange between tristetraprolin and HuR regulates AU-rich element-dependent translation.* PLoS Genet, 2012. **8**(9): p. e1002977.
118. Popp, M.W. and L.E. Maquat, *Organizing principles of mammalian nonsense-mediated mRNA decay.* Annu Rev Genet, 2013. **47**: p. 139-65.
119. Loh, B., S. Jonas, and E. Izaurralde, *The SMG5-SMG7 heterodimer directly recruits the CCR4-NOT deadenylase complex to mRNAs containing nonsense codons via interaction with POP2.* Genes Dev, 2013. **27**(19): p. 2125-38.
120. Ikeda, S., et al., *Altered microRNA expression in human heart disease.* Physiol Genomics, 2007. **31**(3): p. 367-73.
121. Latronico, M.V., D. Catalucci, and G. Condorelli, *Emerging role of microRNAs in cardiovascular biology.* Circ Res, 2007. **101**(12): p. 1225-36.
122. Latronico, M.V. and G. Condorelli, *MicroRNAs and cardiac pathology.* Nat Rev Cardiol, 2009. **6**(6): p. 419-29.
123. Garzon, R. and C.M. Croce, *MicroRNAs in normal and malignant hematopoiesis.* Curr Opin Hematol, 2008. **15**(4): p. 352-8.
124. Kai, Z.S. and A.E. Pasquinelli, *MicroRNA assassins: factors that regulate the disappearance of miRNAs.* Nat Struct Mol Biol, 2010. **17**(1): p. 5-10.
125. Huang, X.A. and H. Lin, *The miRNA Regulation of Stem Cells.* Wiley Interdiscip Rev Membr Transp Signal, 2012. **1**(1): p. 83-95.
126. Altuvia, Y., et al., *Clustering and conservation patterns of human microRNAs.* Nucleic Acids Res, 2005. **33**(8): p. 2697-706.
127. Lee, Y., et al., *MicroRNA genes are transcribed by RNA polymerase II.* EMBO J, 2004. **23**(20): p. 4051-60.
128. Fazi, F. and C. Nervi, *MicroRNA: basic mechanisms and transcriptional regulatory networks for cell fate determination.* Cardiovasc Res, 2008. **79**(4): p. 553-61.
129. Borchert, G.M., W. Lanier, and B.L. Davidson, *RNA polymerase III transcribes human microRNAs.* Nat Struct Mol Biol, 2006. **13**(12): p. 1097-101.
130. Lee, Y., et al., *The nuclear RNase III Drosha initiates microRNA processing.* Nature, 2003. **425**(6956): p. 415-9.
131. Winter, J., et al., *Many roads to maturity: microRNA biogenesis pathways and their regulation.* Nat Cell Biol, 2009. **11**(3): p. 228-34.
132. Matranga, C., et al., *Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes.* Cell, 2005. **123**(4): p. 607-20.
133. Rand, T.A., et al., *Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation.* Cell, 2005. **123**(4): p. 621-9.
134. Lytle, J.R., T.A. Yario, and J.A. Steitz, *Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR.* Proc Natl Acad Sci U S A, 2007. **104**(23): p. 9667-72.
135. Eulalio, A., E. Huntzinger, and E. Izaurralde, *Getting to the root of miRNA-mediated gene silencing.* Cell, 2008. **132**(1): p. 9-14.
136. Bhatt, K., Q.S. Mi, and Z. Dong, *microRNAs in kidneys: biogenesis, regulation, and pathophysiological roles.* Am J Physiol Renal Physiol, 2011. **300**(3): p. F602-10.

137. Berezikov, E., et al., *Mammalian mirtron genes.* Mol Cell, 2007. **28**(2): p. 328-36.
138. Lin, S.L., J.D. Miller, and S.Y. Ying, *Intronic microRNA (miRNA).* J Biomed Biotechnol, 2006. **2006**(4): p. 26818.
139. Lin, S.L. and S.Y. Ying, *Gene silencing in vitro and in vivo using intronic microRNAs.* Methods Mol Biol, 2013. **936**: p. 209-29.
140. Saito, Y., et al., *Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells.* Cancer Cell, 2006. **9**(6): p. 435-43.
141. Scadden, A.D., *The RISC subunit Tudor-SN binds to hyper-edited double-stranded RNA and promotes its cleavage.* Nat Struct Mol Biol, 2005. **12**(6): p. 489-96.
142. Yang, W., et al., *Modulation of microRNA processing and expression through RNA editing by ADAR deaminases.* Nat Struct Mol Biol, 2006. **13**(1): p. 13-21.
143. Kawahara, Y., et al., *Frequency and fate of microRNA editing in human brain.* Nucleic Acids Res, 2008. **36**(16): p. 5270-80.
144. Mitchell, P.S., et al., *Circulating microRNAs as stable blood-based markers for cancer detection.* Proc Natl Acad Sci U S A, 2008. **105**(30): p. 10513-8.
145. Hamilton, A.J., *MicroRNA in erythrocytes.* Biochem Soc Trans, 2010. **38**(Pt 1): p. 229-31.
146. Creemers, E.E., A.J. Tijsen, and Y.M. Pinto, *Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease?* Circ Res, 2012. **110**(3): p. 483-95.
147. Li, C., et al., *Circulating microRNAs as novel and sensitive biomarkers of acute myocardial Infarction.* Clin Biochem, 2012. **45**(10-11): p. 727-32.
148. Wang, K., et al., *Export of microRNAs and microRNA-protective protein by mammalian cells.* Nucleic Acids Res, 2010. **38**(20): p. 7248-59.
149. Kinet, V., et al., *Cardiovascular extracellular microRNAs: emerging diagnostic markers and mechanisms of cell-to-cell RNA communication.* Front Genet, 2013. **4**: p. 214.

Chapter 2: Identification of tristetraprolin (TTP) as a factor that modulates the stability of *CPB2* transcript via binding to the 3'-untranslated region [a]

2.1 Introduction

Appropriate regulation of the balance between coagulation and fibrinolysis is crucial for normal hemostasis [1,2]. Derangement of this balance can result in pathological bleeding or thrombosis. Coagulation and fibrinolysis are tightly regulated at the level of function through modulation and localization of enzyme activity. Both the coagulation and fibrinolytic cascades are a series of zymogen to enzyme conversions that feature positive and negative feedback. Moreover, enzymatic reactions are confined to precise locations though the formation of catalytic complexes on the surface of platelets and fibrin and the presence of high concentrations of fast-acting serpin inhibitors in plasma. A more global form of regulation is achieved through modulation of the expression of genes encoding coagulation and fibrinolytic factors. The genes encoding several coagulation factors, fibrinogen chains, and fibrinolytic inhibitors have been shown to be modulated by inflammatory cytokines as part of the acute phase response [3,4]. Our own work has focused on regulation of the gene encoding thrombin-activatable fibrinolysis inhibitor (TAFI), a plasma protein that may provide mechanistic links between coagulation and fibrinolysis and between coagulation and inflammation [5,6].

Proteolytic cleavage of TAFI by thrombin, the thrombin-thrombomodulin complex, or plasmin results in the formation of an enzyme (TAFIa) with basic carboxypeptidase activity [7-9]. TAFIa downregulates fibrinolysis by removing the carboxyl-terminal lysine residues from partially degraded fibrin that mediate positive feedback in the fibrinolytic cascade [10]. TAFIa also inactivates several pro-inflammatory peptides and proteins containing carboxyl-terminal basic residues including

[a] This is the outcome of joint research

bradykinin, the anaphylatoxins C3a and C5a, thrombin-cleaved osteopontin, and plasmin-cleaved chemerin [11,12]. Substantial variation in plasma TAFI concentrations has been observed [5]. Elevated plasma TAFI concentrations have been associated with risk for both arterial and venous thrombotic events [13-21]. Approximately 25% of the total variation in plasma TAFI concentrations has been attributed to genetic factors, and many single nucleotide polymorphisms (SNPs) in *CPB*2, the gene encoding TAFI, have been identified [22,23]. The three SNPs in the 3'-untranslated region (3'-UTR) of the *CPB2* mRNA have been shown to affect mRNA stability, indicating a potential role for these SNPs in influencing *CPB2* mRNA abundance and hence TAFI synthesis by the liver [24]. In accordance with these findings, we have previously determined a key role for the 3'-UTR in mediating *CPB2* mRNA stability both in the steady state and in response to inflammatory cytokines [25]. In the current study, we set out to identify the sequences in the 3'-UTR that mediate this control of mRNA stability and to identify protein factors that may bind to these sequences.

2.2 Experimental Procedures

2.2.1.Plasmid Constructions

Restriction and modifying enzymes were purchased from Stratagene, New England Biolabs, or Invitrogen. Fusion mRNA reporter plasmids were constructed using the expression vector pC7βG described by Wilson and Deeley [26]. A series of 5'-deletions of the *CPB*2 3'-UTR (see Fig. 1), each approximately 50 nucleotides long starting from the stop codon, were constructed by PCR. The 3' boundary of each PCR product was the 5'-most polyadenylation site at nucleotide +1660 [27]. The primers contained cryptic *Pac*I sites for insertion of the PCR products into the *Pac*I site of pC7βG. Two different internal deletions in the 3'-UTR (see Fig. 1) were also constructed

by overlap PCR. The deletions removed nucleotides +1541 to +1668 (ΔP1) or +1667 to +1801 (ΔP2). The 3' boundary of both internal deletions was the 3'-most polyadenylation site at +1819. The outside primers for the respective overlap PCR schemes contained cryptic *Pac*I sites for insertion of the PCR products into pC7βG. In all cases, PCR products were first cloned into pBluescript II SK+ (Stratagene) for sequence analysis, and were then excised with *Pac*I digestion for insertion into pC7βG.

Site-directed mutagenesis of the parental plasmid βG-*CPB2*/1273-1819 [25] was accomplished using the QuikChange XL kit (Stratagene). The sense mutagenic primer was as follows: 5' – GAT TTC TGC TCC AAA TTT TCA AGA AAG GGC TGC TTG TGC CTT TAG AAA TAC – 3'. The underlined bases are mutations from T to G aimed at abolishing the putative tristetraprolin (TTP) binding site. The presence of the mutation was verified by DNA sequence analysis.

2.2.2 Cell Culture

HepG2 (human hepatocellular carcinoma) cells were grown in minimum essential medium (MEM) (Invitrogen) containing 10% fetal bovine serum (FBS; American Type Culture Collection) and 1% (v/v) penicillin-streptomycin-Fungizone (PSF; Invitrogen). The cells were maintained at 37°C in a humidified atmosphere consisting of 95% room air/5% CO2. Cells were stably transfected with β-globin fusion plasmids as previously described [25] and were thereafter maintained in the presence of 300 μg/mL hygromycin B (Roche).

2.2.3 mRNA Decay Assay and RNA Isolation

Stably transfected HepG2 cell lines were seeded in 6-well plates or 100 mm plates and grown to 90% confluence. Actinomycin D (Unites States Biochemical or Sigma-Aldrich) was added to each well at a final concentration of 5 μg/mL. Total RNA was

harvested 0, 1, 2, 4, 6 or 8 hours after the addition of actinomycin D using TriZOL reagent (Invitrogen) or RNeasy Mini Kits (QIAGEN) as directed by the manufacturer.

2.2.4 Northern Blot Analysis

Northern blot analysis was performed, using 10 μg/lane total RNA, as previously described [25]. Detection of the β-globin fusion transcripts or glyceraldehyde-6-phosphate dehydrogenase (GAPDH) transcripts (as a control for RNA loading and transfer) was performed with by hybridization with ^{32}P-labeled DNA probes followed by exposure to a phosphor screen or with digoxigenin-labeled antisense RNA probes followed by detection using anti-digoxigenin antibodies and chemiluminescence (kit from Roche). The fraction of β-globin signal remaining at each time point was determined following correction for the amount of GAPDH transcript present, which was itself corrected by taking into account the half-life of GAPDH mRNA (8 hours) [25].

2.2.5 RNA Electrophoretic Mobility Shift Assay

The RNA probes employed are depicted in Table 1. A mutant version of probe RNA-3a was made by substituting the underlined nucleotides with G nucleotides. The corresponding DNA sequences were purchased as overlapping oligonucleotides that were annealed and then extended using T4 DNA polymerase. The resulting fragments were inserted into pBluescript II SK+ at the *Eco*RV site. The plasmids were then linearized with *Hind*III prior to *in vitro* transcription in the presence of [α-^{32}P]-CTP (Perkin Elmer) using T3 polymerase and the Riboprobe *In Vitro* Transcription kit (Promega). Cytoplasmic extracts were prepared from confluent HepG2 cells by sonication of scraped cells for 1 min in 10 mM HEPES pH 7.9, 1.5 mM MgCl2, 10 mM KCl, containing protease inhibitor cocktail (Sigma) followed by pelleting of cell debris for 5 min at 12,000 × g. Approximately 2 × 105 cpm of RNA probe was incubated with or without 10

μg of cytoplasmic protein extract in incubation buffer (5 mM HEPES pH 7.9, 0.5 mM MgCl2, 7.5 mM KCl, 0.5 mM DTT, 0.12 mM EDTA) in a final volume of 20 μL for 30 min at room temperature. In some cases, 0.2 μg of goat polyclonal anti-TTP antibodies (Santa Cruz Biotechnology) were added to some of the reaction mixtures and incubation of all reactions was continues for a further 1 hour on ice. Reactions were then treated with 1 μg/mL RNase A for 10 minutes at 37°C prior to electrophoresis on non-denaturing 6% polyacylamide gels in a buffer system consisting of 22 mM Tris-HCl pH 8.0, 22 mM borate, 5 mM EDTA. Gels were dried and exposed to Kodak X-OMAT film.

2.3 Results

2.3.1 Identification of instability elements in the CPB2 3'-UTR

A β-globin fusion mRNA reporter system was used to identify cis-acting elements in the *CPB2* 3'-UTR that modulate *CPB2* mRNA stability. The results of our previous study using this system clearly imply the presence of *cis*-acting elements in the *CPB2* 3'-UTR that control the stability and thus abundance of this transcript. In order to identify these elements, *CPB2* 3'-UTR sequences were inserted downstream of the rabbit β-globin sequence in the pC7βG plasmid (see Fig. 2.2) that ended at the 5'-most polyadenylation site and that represented progressive 5'-deletions starting approximately 100 nucleotides downstream of the stop codon (see Fig. 2.1). HepG2 cell lines stably expressing the respective fusion mRNAs were treated with actinomycin D to arrest transcription and total RNA was harvested at different time points. The amount of fusion transcript remaining at each time point was measured by Northern blot analysis using a β-globin cDNA probe and the half-life of the respective fusion transcripts was determined from the resultant decay curves (Fig. 2.2 and Table 2.2). The data indicate that deletion of the first 150 nucleotides of the 3'-UTR has no effect on the stability of the fusion transcripts, as

they are very similar to that of the parental fusion transcript containing the entire 3'-UTR up to the first polyadenylation site. Deletion of a further 50 nucleotides (Δ208 construct) results in a fusion mRNA with reduced stability, indicating that the sequence between +1424 and +1482 harbors a stability element. Conversely, deletion of a further 50 nucleotides (Δ250 construct) results in a fusion mRNA with an enhanced stability, indicating that the sequence between +1481 and +1523 harbors an instability element.

There are three possible polyadenylation sites in the *CPB2* 3'-UTR; inclusion of sequences downstream of the first and second sites results in a progressive destabilization of the *CPB2* mRNA, indicating the presence of *cis*-acting instability elements in these regions. Accordingly, we constructed β-globin fusion plasmids containing deletions of sequences upstream of the second and third polyadenylation sites (ΔP1 and ΔP2, respectively; Figs. 2.1 and 2.3). The stabilities of the respective transcripts were substantially increased relative to their parental constructs (Fig. 2.3 and Table 2.2) indicating the presence of instability elements in both of these regions.

```
                                                                                    →Δ114
+1270  TAATGCCCCT GATTTTATCA TTCTGCTTCC GTATTTTAAT TTACTGATTC CAGCAAGACC
+1330  AAATCATTGT ATCAAATTAT TTTTAAGTTT TATCCGTAGT TTTGATAAAA GATTTTCCTA
                                            →Δ150
+1390  TTCCTTGGTT CTGTCAGAGA ACCTAATAAG TGCTACTTTG CCATTAAGGC AGACTAGGGT
                                          →Δ208
+1450  TCATGTCTTT TTACCCTTTA AAAAAAATTG TAAAAGTCTA GTTACCTACT TTTTCTTTGA
                    →Δ249
+1510  TTTTCGACGT TTGACTAGCC ATCTCAAGCA AGTTTCGACG TTTGACTAGC CATCTCAAGC
+1570  AAGTTTAATC AAAGATCATC TCACGCTGAT CATTGGATCC TACTCAACAA AAGGAAGGGT
+1630  GGTCAGAAGT ACATTAAAGA TTTCTGCTCC AAATTTTCAA TAAATTTCTG CTTGTGCCTT
+1690  TAGAAATACA ACCATGCATT CCGTTTGCTC CACGGTAATT AGGCGATGGC CCAGAAAGGG
+1750  GAGGGGTGTC AAAAACGACA AACATAGCCT CTCATTCCAG CTCAGCTGCT CAATAAACAC
+1810  TGTTGAACG
```

Figure. 2.1 Topology of human *CPB2* 3'-UTR and location of deletion mutants. Shown is the cDNA sequence between the stop codon (double-underlined) and including all three potential polyadenylation sites (curved arrows). Putative polyadenylation signal sequences are boxed. The 5'-boundaries of the 5' deletion variants are indicated with the bent arrows; the 3'-boundary of these variants is the first polyadenylation site at +1660. The sequences deleted in the ΔP1 and ΔP2 variants are indicated with the dashed and dotted lines, respectively; the 3'-boundary of these variants is the last polyadenylation site at +1819. All cDNA fragments contained *Pac*I sites on either end for insertion into the episomal β-globin fusion mRNA vector pC7βG.

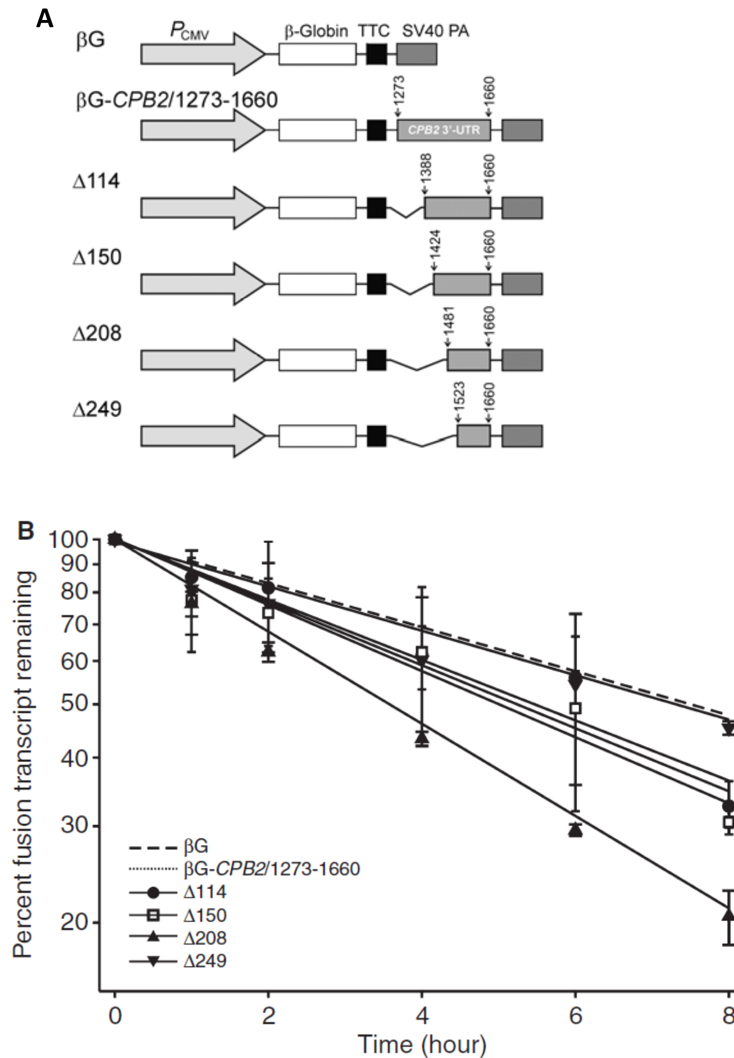

Figure 2.2 5'-deletion analysis of the *CPB2* 3'-UTR. Panel A. Schematic representation of the β-globin reporter plasmid constructs expressing fusion mRNA species. All variants contained the cytomegalovirus promoter (P_{CMV}), a segment of rabbit β-globin cDNA, a translation termination cassette (TTC), and the SV40 polyadenylation signal sequence. Boundaries of the *CPB2* insert are indicated, with nucleotide numbering corresponding to that in ref. 25. Fusion transcripts contained β-globin and *CPB2* 3'-UTR segments. Panel B. HepG2 cells stably transfected with the β-globin reporter plasmids were treated with actinomycin D (5 μg/mL); RNA was harvested at various times after the addition of the drug. The amounts of the respective fusion transcripts were determined by Northern blot analysis using a probe specific for rabbit β-globin cDNA and using a probe specific for the GAPDH mRNA as an internal standard to account for differences in RNA loading and transfer. Fusion mRNA abundance after addition of actinomycin D is shown relative to the amount present immediately before addition of the drug. The data are the means of three independent experiments; data for βG and βG-*CPB2*/1273-1660 are from ref. 25.

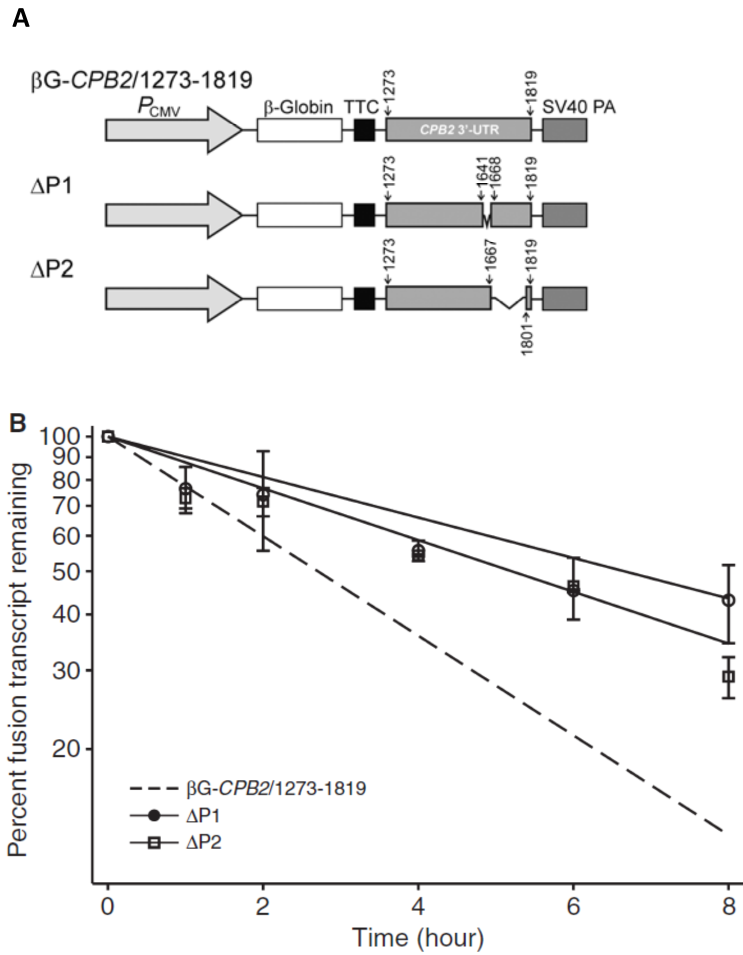

Figure 2.3 Internal deletion analysis of the *CPB2* 3'-UTR. Panel A. Schematic representation of the β-globin reporter plasmid constructs expressing fusion mRNA species. Features of the respective constructs are as described in the Legend to Fig. 2.2 Panel B. The intrinsic stabilities of each mRNA species were determined as described in the Legend to Fig. 2. The data are the means of three independent experiments; data for βG-*CPB2*/1273-1819 are from ref. 25.

2.3.2 Detection of trans-acting factors binding to instability elements

The presence of *cis*-acting elements in the *CPB2* 3'-UTR suggests that *trans*-acting factors such as cytoplasmic proteins associate with these elements to modulate *CPB2* mRNA stability. Accordingly, a series of radiolabeled single stranded RNA probes were prepared (Table 2.1) corresponding to (i) the instability element located between +1482 and +1523 (RNA-1); (ii) the instability element located between the first and second polyadenylation sites (RNA-2); and (iii) the instability element located between the second and third polyadenylation sites. Owing to the long length of this last region, three overlapping RNA probes were synthesized (RNA-3a,b,c). The probes were incubated with HepG2 cytoplasmic protein extracts and the mixtures were then subjected to RNase A treatment followed by non-denaturing polyacrylamide gel electrophoresis to identify protein-RNA complexes.

No detectable protein-RNA complexes were observed for probes RNA-1, RNA-3b, or RNA-3c (data not shown). However, a clearly detectable band of reduced electrophoretic mobility was observed after RNase A treatment for probes RNA-2 and RNA-3a (Figs. 2.4 and 2.5). A search of the database of protein-binding sequences in RNA revealed the presence of a putative binding site for tristetraprolin (TTP) [28] within the RNA-3a sequence (Table 2.1). Accordingly, we performed an RNA mobility shift assay in the presence of RNA-3a, cytoplasmic extract, and an antibody specific for human TTP. Under these conditions, an additional band of lower mobility is observed (open arrow, Fig. 2.5) which presumably corresponds to a ternary complex between the probe, TTP, and the antibody.

To verify the role of the putative TTP binding site in RNA-3a in binding to cytoplasmic factors, we prepared a radiolabeled RNA probe in which nucleotides within

the consensus TTP binding site were mutated (Table 2.1). This probe failed to bind to cytoplasmic proteins (Fig. 2.6). Taken together, the data in Figs. 2.5 and 2.6 strongly indicate that nucleotides +1668 to +1677 are capable of binding to TTP.

In order to directly assess the role of TTP in modulating *CPB2* mRNA stability, the mutation in the putative TTP binding site was introduced into the β-globin fusion mRNA reporter plasmid βG-*CPB2*/1273-1819; we then examined the stability of the resultant transcript using actinomycin D treatment of stably expressing HepG2 cells and Northern blot analysis using a DIG-labeled riboprobe. Mutation of the putative TTP binding site resulted in a substantial increase in fusion transcript stability (Fig. 2.7 and Table 2.2), in keeping with the role of TTP as an mRNA instability factor.

Table 2.1 Sequences of probes used in RNA mobility shift assays

RNA	Nucleotides	Sequence (5' – 3')
1	+1470 to +1529	AAAAAAAUUG UAAAAGUCUA GUUACCUACU UUUUCUUUGA UUUUCGACGU UUGACUAGCC
2	+1641 to +1667	AUUAAAGAUU UCUGCUCCAA AUUUUC
3	+1660 to +1719	a) AAAUUUUC**AA <u>U</u>AAA<u>UUU</u>**CUG CUUGUGCCUU UAGAAAUACA ACCAUGCAUU CCGUUUGCUC[a]
	+1700 to +1759	b) ACCAUGCAUU CCGUUUGCUC CACGGUAAUU AGGCGAUGGC CCAGAAAGGG GAGGGGUGUC
	+1740 to +1800	c) CCAGAAAGGG GAGGGGUGUC AAAAACGACA AACAUAGCCU CUCAUUCCAG CUCAGCUGCU C

[a] the consensus TTP binding site is shown in boldface type; the underlined nucleotides in probe RNA-3a were mutated to G in probe RNA-3aΔTTP

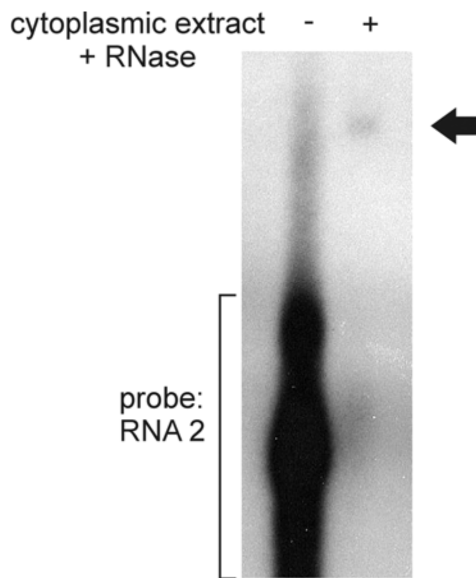

Figure 2.4 Detection of a cytoplasmic protein bound to an RNA sequence corresponding to the region between the first and second polyadenylation sites. A radiolabeled probe encompassing this region (designated RNA 2 in Table 2.1) was synthesized by *in vitro* transcription from a plasmid template. The probe was incubated in the presence of cytoplasmic extract prepared from HepG2 cells, followed by treatment of the binding reaction with RNase A. A control reaction lacked both cytoplasmic extract and RNase A treatment. Reactions were subjected to non-denaturing polyacrylamide gel electrophoresis and radiolabeled RNA complexes were visualized by autoradiography. The positions of migration of free RNA 2 probe and the protein/RNA complex of lower mobility (arrow) are indicated.

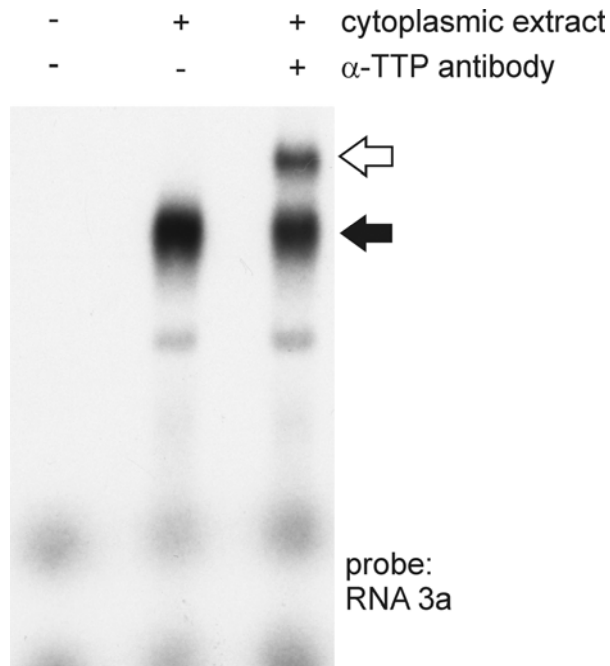

Figure 2.5 Detection of binding of TTP to an RNA sequence within a region between the second and third polyadenylation sites. A radiolabeled probe encompassing the first third of this region (designated RNA 3a in Table 2.1) was synthesized by *in vitro* transcription from a plasmid template. The probe was incubated in the presence of cytoplasmic extract prepared from HepG2 cells either in the presence or absence of a polyclonal anti-TTP antibody, followed by treatment of the binding reaction with RNase A. A control reaction lacked cytoplasmic extract. Reactions were subjected to non-denaturing polyacrylamide gel electrophoresis and radiolabeled RNA complexes were visualized by autoradiography. The positions of migration of free RNA 3a probe, a protein/RNA complex of lower mobility (filled arrow), and a supershifted complex observed in the presence of antibody (open arrow) are indicated.

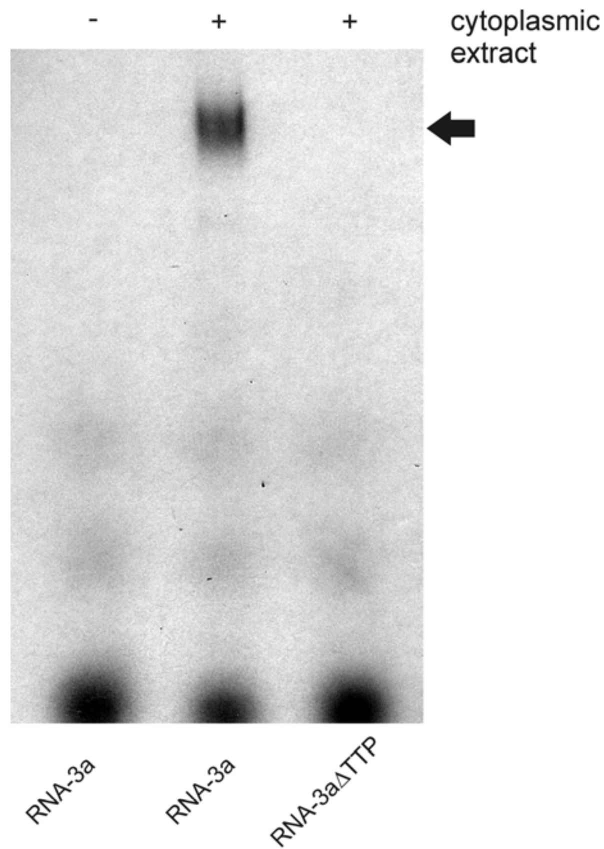

Figure 2.6 Mutagenesis of the putative TTP binding site in RNA 3a abolishes TTP binding. Radiolabeled RNA 3a probe or a mutant version in which nucleotides necessary for TTP binding were mutated as indicated in Table 2.1 was incubated with cytoplasmic extract prepared from HepG2 cells, followed by treatment of the binding reaction with RNase A. A control reaction lacked cytoplasmic extract. Reactions were subjected to non-denaturing polyacrylamide gel electrophoresis and radiolabeled RNA complexes were visualized by autoradiography. The positions of migration of free RNA 3a probe and a protein/RNA complex of lower mobility (arrow) are indicated.

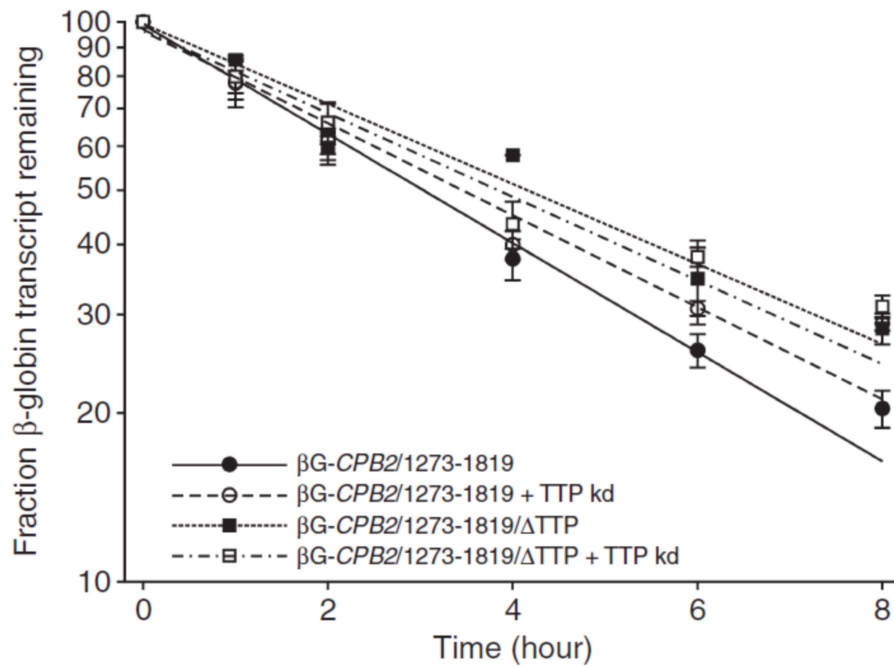

Figure 2.7 Mutagenesis of the putative TTP site in the *CPB2* 3'-UTR stabilizes a β-globin fusion transcript. The ΔTTP mutations indicated in Table 1 were introduced into the βG-*CPB2*/1273-1819 fusion mRNA reporter plasmid. The intrinsic stabilities of the wild-type and mutant fusion mRNA species were determined as described in the Legend to Fig. 2.2. The data are the means of three independent experiments.

Table 2.2 Half-lives of fusion transcripts

Fusion mRNA construct	$t_{1/2}$ (hrs)[a]
βG	~7.5[b]
βG-*CPB2*/1273-1660	~5.0[a]
Δ114	5.5 ± 0.3
Δ150	5.2 ± 0.4
Δ208	3.6 ± 0.2
Δ249	7.4 ± 0.8
βG-*CPB2*/1273-1819	~2.6[b]
ΔP1	5.2 ± 0.2
ΔP2	6.7 ± 0.4
ΔTTP	6.4 ± 0.2

[a] Data shown are the means ± s.e.m. of three independent experiments
[b] Data are from ref. 25

2.4 Discussion

In this study we sought to identify *cis*-elements within *CPB2* transcript 3'UTR and *trans*-acting factors that bind to the respective sequences and participate in modulation of *CPB2* transcript stability. We have identified one stability element and three stability elements within the 3'-UTR; two of the stability elements were shown to bind to cytoplasmic proteins using RNA gel mobility shift analyses, and one of these binding factors was identified as TTP. Our data are the first to explore the factors modulating *CPB2* mRNA stability, and have implications for the determination of steady-state *CPB2* mRNA levels as well as for the regulation of *CPB2* mRNA stability by inflammatory mediators.

The role of TTP in modulation of mRNA stability was uncovered more than a decade ago by studies in TTP knockout mice that exhibited generalized inflammation as a result of overproduction and accumulation of the pro-inflammatory cytokine TNFα [29]. TTP was found to specifically bind the AU-rich element (ARE) in the 3'-UTR of TNFα transcript causing rapid degradation via ARE-mediated decay (AMD) [30,31]. Targets of TTP uncovered so far include mainly cytokines and proto-oncogenes, but also include plasminogen activator inhibitor-2 [32,33]. However, mRNAs associate with many RNA-binding proteins thus creating an mRNP (messenger ribonucleoprotein); as such, the regulatory role of TTP with some of these targets may be influenced by some other factor(s) in the mRNP and thus are difficult to predict from the mere presence of TTP.

Of the many targets that have been identified for TTP so far, most code for proteins involved in inflammation. We have now identified TTP as a key regulator of *CPB2* mRNA stability, thereby expanding the pool of transcripts regulated by TTP outside of the cytokine family. Our own work has previously shown that *CPB2* transcript

stability is modulated by inflammatory cytokines IL-1β and IL-6 [25]. Therefore, cytokines/TTP/TAFI mRNA axis may be instrumental in integration of TAFI within the inflammatory response, as it appears to undergo regulation by factors common to other cytokines, such as TNFα and GM-CSF. Although it is tempting to speculate that TAFI levels would be elevated in TTP-/- mice, we observed that TNFα treatment of cultured HepG2 cells had a destabilizing effect on the *CPB2* transcripts (data not shown).

In the present study we have identified a stability element residing upstream of the first polyadenylation site of the *CPB2* 3'UTR. However, the identity of the *trans*-acting factor binding to it is presently unknown, as well as that of the factor(s) binding to the two other instability Identification of these *trans*-acting factors is necessary, as they do not function in isolation. It would be intriguing to discover whether these RNA binding factors are shared with those of cytokines, which would further support the role of TAFI as an integral part of the inflammatory response. Additionally, we have previously shown that polyadenylation site selection within the *CPB2* transcript is modulated by cytokines, whereby the longest transcript is preferentially formed over the other two in the presence of IL-1β and IL-6 and is the least stable [25]. Curiously, it is the only form that contains the TTP binding site. The sequences that modulate polyadenylation site selection within the *CPB2* 3'-UTR have not been identified so far. It has been observed for other transcripts containing tandem signals like the *CPB2* transcript that the most upstream signal is the most frequently used [36-39]. In addition, the distance between the signals also plays a role, whereby a stronger downstream signal can out-compete a weaker upstream signal if the two signals are less than 400 base pairs apart [35]. Therefore, preferential formation of the longest transcript that has the lowest half-life in the presence of acute phase mediators points to the role of TTP as a very potent modulator of *CPB2*

mRNA stability under inflammatory conditions, not only as an instability factor but as a factor involved in polyadenylation site selection. Future work is required to identify other RNA proteins that bind to the stability/instability *cis*-elements we discovered in the current study in order to derive a mechanism of *CPB2* transcript regulation. More importantly, these studies would uncover major players in the maintenance of the steady state abundance of the *CPB2* transcripts and scenarios in which their imbalance would lead to the pathological phenotypes.

2.5 References

1. Nesheim M, Wang W, Boffa M, Nagashima M, Morser J, Bajzar L. *Thrombin, thrombomodulin and TAFI in the molecular link between coagulation and fibrinolysis.* Thromb Haemost 1997; 78: 386-91.
2. Nesheim M. *Thrombin and fibrinolysis*. Chest 2003; 124 (Suppl 3): 33S-9S.
3. Levi M, van der Poll T. *Two-way interactions between inflammation and coagulation.* Trends Cardiovasc Med 2005; 15: 254-9.
4. Koenig W. *Fibrin(ogen) in cardiovascular disease: an update*. Thromb Haemost
    a. 2003; 89: 601-9.
5. Boffa MB, Koschinsky ML. *Curiouser and curiouser: Recent advances in measurement of thrombin-activatable fibrinolysis inhibitor (TAFI) and in understanding its molecular genetics, gene regulation, and biological roles*. Clin Biochem 2007; 40: 431-42.
6. Morser J, Gabazza EC, Myles T, Leung LL. *What has been learnt from the thrombin-activatable fibrinolysis inhibitor-deficient mouse*? J Thromb Haemost 2010; 8: 868-76
7. Bajzar L, Manuel R, Nesheim ME. *Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor*. J Biol Chem 1995; 270: 14477-84.
8. Bajzar L, Morser J, Nesheim M. *TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex*. J Biol Chem 1996; 271: 16603-8.
9. Mao SS, Cooper CM, Wood T, Shafer JA, Gardell SJ. *Characterization of plasmin-mediated activation of plasma procarboxypeptidase B*. Modulation by glycosaminoglycans. J Biol Chem 1999; 274: p.35046-52.
10. Wang W, Boffa MB, Bajzar L, Walker JB, Nesheim ME. *A study of the mechanism of inhibition of fibrinolysis by activated thrombin-activable fibrinolysis inhibitor.* J Biol Chem 1998; 273:p.27176-81.
11. Leung LL, Nishimura T, Myles T. *Regulation of tissue inflammation by thrombin-activatable carboxypeptidase B (or TAFI)*. Adv Exp Med Biol 2008; 632:p. 61-9.
12. Du XY, Zabel BA, Myles T, Allen SJ, Handel TM, Lee PP, Butcher EC, Leung LL. *Regulation of chemerin bioactivity by plasma carboxypeptidase N, carboxypeptidase B (activated thrombin-activable fibrinolysis inhibitor), and platelets.* J Biol Chem 2009; 284:p.751-8.
13. van Tilburg NH, Rosendaal FR, Bertina RM. *Thrombin activatable fibrinolysis inhibitor and the risk for deep vein thrombosis.* Blood 2000; 95: p.2855-9.
14. Meltzer ME, Lisman T, de Groot PG, Meijers JC, le Cessie S, Doggen CJ, Rosendaal FR. *Venous thrombosis risk associated with plasma hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1*. Blood 2010; 116: p.113-21.
15. Eichinger S, Schönauer V, Weltermann A, Minar E, Bialonczyk C, Hirschl M, Schneider B, Quehenberger P, Kyrle PA. *Thrombin-activatable fibrinolysis inhibitor and the risk for recurrent venous thromboembolism.* Blood 2004; 103: p.3773-6.
16. Leebeek FW, Goor MP, Guimarães AH, Brouwers GJ, Maat MP, Dippel DW, Rijken DC. *High functional levels of thrombin-activatable fibrinolysis inhibitor*

are associated with an increased risk of first ischemic stroke. J Thromb Haemost 2005; 3: p.2211-8.

17. Santamaría A, Oliver A, Borrell M, Mateo J, Belvis R, Martí-Fábregas J, Ortín R, Tirado I, Souto JC, Fontcuberta J. *Risk of ischemic stroke associated with functional thrombin-activatable fibrinolysis inhibitor plasma levels*. Stroke 2003; 34:p. 2387-91.
18. Rooth E, Wallen H, Antovic A, von Arbin M, Kaponides G, Wahlgren N, Blombäck M, Antovic J. *Thrombin activatable fibrinolysis inhibitor and its relationship to fibrinolysis and inflammation during the acute and convalescent phase of ischemic stroke.* Blood Coag Fibrinolysis 2007; 18:p. 365-70.
19. Ladenvall C, Gils A, Jood K, Blomstrand C, Declerck PJ, Jern C. *Thrombin activatable fibrinolysis inhibitor activation peptide shows association with all major subtypes of ischemic stroke and with TAFI gene variation.* Arterioscler Thromb Vasc Biol 2007; 27:p. 955-62.
20. de Bruijne EL, Gils A, Guimarães AH, Dippel DW, Deckers JW, van den Meiracker AH, Poldermans D, Rijken DC, Declerck PJ, de Maat MP, Leebeek FW. *The role of thrombin activatable fibrinolysis inhibitor in arterial thrombosis at a young age: the ATTAC study.* J Thromb Haemost 2009; 7: p.919-27.
21. Trégouet DA, Schnabel R, Alessi MC, Godefroy T, Declerck PJ, Nicaud V, Munzel T, Bickel C, Rupprecht HJ, Lubos E, Zeller T, Juhan-Vague I, Blankenberg S, Tiret L, Morange PE; AtheroGene Investigators. *Activated thrombin activatable fibrnolysis inhibitor levels are associated with the risk of cardiovascular death in patients with coronary artery disease: the Athero Gene study.* J Thromb Haemost 2009; 7: p.49-57.
22. Frère C, Morange PE, Saut N, Trégouet DA, Grosley M, Beltran J, Juhan-Vague I, Alessi MC. *Quantification of thrombin activatable fibrinolysis inhibitor (TAFI) gene polymorphism effects on plasma levels of TAFI measured with assays insensitive to isoform-dependent artefact*. Thromb Haemost 2005; 94: p.373-9.
23. Frère C, Trégouet DA, Morange PE, Saut N, Kouassi D, Juhan-Vague I, Tiret L, Alessi MC. *Fine mapping of quantitative trait nucleotides underlying thrombin-activatable fibrinolysis inhibitor antigen levels by a transethnic study*. Blood 2006; 108: p.1562-8.
24. Boffa MB, Maret D, Hamill JD, Bastajian N, Crainich P, Jenny NS, Tang Z, Macy EM, Tracy RP, Franco RF, Nesheim ME, Koschinsky ML. *Effect of single nucleotide polymorphisms on expression of the gene encoding thrombin-activatable fibrinolysis inhibitor: a functional analysis.* Blood 2008; 111:p. 183-9.
25. Maret D, Boffa MB, Brien DF, Nesheim ME, Koschinsky ML. *Role of mRNA transcript stability in modulation of expression of the gene encoding thrombin activable fibrinolysis inhibitor.* J Thromb Haemost 2004; 2: p.1969-79.
26. Wilson GM, Deeley RG. *An episomal expression vector system for monitoring sequence-specific effects on mRNA stability in human cell lines.* Plasmid 2005; 33: p.198-207.
27. Boffa MB, Reid TS, Joo E, Nesheim ME, Koschinsky ML. *Characterization of the gene encoding human TAFI (thrombin-activable fibrinolysis inhibitor; plasma procarboxypeptidase B.* Biochemistry 1999; 38: p.6547-58.

28. Carrick DM, Lai WS, Blackshear PJ. *The tandem CCCH zinc finger protein tristetraprolin and its relevance to cytokine mRNA turnover and arthritis.* Arthritis Res Therapy 2004; 6: p.248-64.
29. Taylor GA, Carballo E, Lee DM, Lai WS, Thompson MJ, Patel DD, Schenkman DI, Gilkeson GS, Broxmeyer HE, Haynes BF, Blackshear PJ. *A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency.* Immunity 1996; 4:p. 445-54.
30. Lai WS, Carballo E, Thorn JM, Kennington EA, Blackshear PJ. *Interactions of CCCH zinc finger proteins with mRNA. Binding of tristetraprolin-related zinc finger proteins to AU-rich elements and destabilization of mRNA.* J Biol Chem 2000; 275:p.17827-37.
31. 31.Stoecklin G, Colombi M, Raineri I, Leuenberger S, Mallaun M, Schmidlin M, Gross B, Lu M, Kitamura T, Moroni C. *Functional cloning of BRF1, a regulator of ARE-dependent mRNA turnover*. EMBO J 2002; 21: p.4709-18.
32. Yu H, Stasinopoulos S, Leedman P, Medcalf RL. *Inherent instability of plasminogen activator inhibitor type 2 mRNA is regulated by tristetraprolin*. J Biol Chem 2003; 278: p.13912-8.
33. Sandler H, Stoecklin G. *Control of mRNA decay by phosphorylation of tristetraprolin.* Biochem Soc Trans 2008; 36:p.491-6
34. Kruithof EK, Baker MS, Bunn CL. *Biological and clinical aspects of plasminogen activator inhibitor type 2.* Blood 1995; 86:p.4007-24.
35. Batt DB, Luo Y, Carmichael GG. *Polyadenylation and transcription termination in gene constructs containing multiple tandem polyadenylation signals*. Nucleic Acids Res 1994; 22: p.2811-6.
36. Tantravahi J, Alvira M, Falck-Pedersen E. *Characterization of the mouse βmaj globin transcription termination region: a spacing sequence is required between the poly(A) signal sequence and multiple downstream termination elements*. Mol Cell Biol 1993; 13: p.578-87.
37. Peterson ML, Perry RP. *Regulated production of μm and μs mRNA requires linkage of the poly(A) addition sites and is dependent on the length of the μs-μm intron.* Proc Natl Acad Sci USA 1986; 83:p. 8883-7.
38. Nishikura K, Vuocolo GA. *Synthesis of two mRNAs by utilization of alternate polyadenylation sites: expression of SV40-mouse immunoglobulin μ chain gene recombinants in Cos monkey cells.* EMBO J. 1984; 3: p.689-99.
39. Denome RM, Cole CN. *Patterns of polyadenylation site selection in gene constructs containing multiple polyadenylation signals.* Mol Cell Biol 1988; 8: p.4829-39.

Chapter 3: Inflammatory cytokines reduce thrombin activatable fibrinolysis inhibitor (TAFI) expression via tristetraprolin-mediated mRNA destabilization

3.1 Introduction

Transcription was long considered to be the deciding factor in regulation of gene expression, but the importance of post-transcriptional regulation continues to gain recognition. Sub-cellular sorting of *de novo* synthesized messenger RNAs (mRNAs) is a well-orchestrated process, and one that is susceptible to regulation at multiple steps. Specific RNA sequences contained primarily in the 3'-untranslated region (3'-UTR) of mRNAs are crucial in determining the fate of the message through the recruitment of *trans*-acting factors that recognize them. The stability of mRNAs harbouring adenylate-uridylate rich elements (AREs) in their 3'- UTRs and thus the level of expression of their protein products is dictated by the effect that ARE-binding proteins exert. Sustained mRNA stabilization can be caused by increased activity of mRNA stabilizing factors, such as human antigen R (HuR, *ELAVL1*) or by reduced activity of mRNA decay-promoting factors, such as tristetraprolin (TTP, *ZFP36*) [1-3].

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a human plasma zymogen that regulates the balance between coagulation and fibrinolysis and that also mediates molecular connections between hemostasis and inflammation [4,5]. The major contribution to the plasma pool of TAFI is accounted for by the expression of the gene encoding it, *CPB2*, in the liver [6,7]. TAFI can be activated by thrombin [8] and plasmin [9], but most efficiently by thrombin in complex with the cofactor thrombomodulin [10]. The activated form of TAFI (TAFIa) acts as a basic carboxypeptidase, targeting substrates such as partially degraded fibrin and carboxyl-terminal lysine residues from cell surface receptors [11], pro-inflammatory modulators (anaphylatoxins C3a and C5a)

[12] , bradykinin [13], thrombin-cleaved osteopontin [14], and the anti-inflammatory modulator, plasmin-cleaved chemerin [15]. The role of TAFI in inflammatory disease is multifaceted and involves both recognition of specific pro-inflammatory substrates by TAFIa [12-15], and the regulation of TAFI gene expression by inflammatory mediators [16]. We have also discovered that macrophages produced by differentiation of the monocytoid cell line THP-1 produce TAFI protein [17], which provides new insights into the extra-vascular, non-fibrinolytic functions of TAFI that take place in the arterial wall, wound fields and the peritoneum.

Our group has published a series of studies characterizing the regulation of expression of the *CPB2* [16-22]. The *CPB2* transcript contains three potential polyadenylation (polyA) sites resulting in transcripts with 3'-UTRs of different lengths [6] and intrinsic stabilities [18]. Additionally, interleukins (IL)-6 and -1β, when administered in combination to HepG2 (human hepatoma) cells, lead to preferential formation of the longest transcript that is further destabilized by a factor of two [16]. This suggests the presence of inducible instability elements, possibly contained within the region between the last two polyA sites.

Most recently, we published a study identifying *cis*-sequences in the *CPB2* 3'-UTR that mediate transcript stability, as well as documenting the effects of *trans*-acting factors binding to them [21]. These studies revealed that TTP binds between the second and the third polyA sites and acts to destabilize *CPB2* mRNA. The aim of the present investigation was two-fold: to determine the identities of other *trans*-acting factors binding to the *CPB2* 3'-UTR and the modulatory effects they put forth on transcriptional and post-transcriptional regulation of *CPB2* gene expression in the presence of inflammatory stimuli.

3.2 Experimental procedures

3.2.1 Plasmid constructions

The β-globin reporter fusion mRNA expression plasmids βG-*CPB2/*3'UTR and βG-*CPB2/*3'UTRΔTTP were derived from previously described plasmids [21] as follows. The pC7βG plasmid that we used was modified by the replacement of the existing cytomegalovirus promoter (CMV) with a doxycycline responsive version of the promoter derived from pTET-BBB [23], which was the kind gift of Dr. Robert Medcalf (Australian Centre for Blood Diseases, Monash University). The promoter and tet operon were excised from pTET-BBB using *Xho*I and *Bbs*I, the ends were made blunt using T4 DNA polymerase and the fragment was inserted into *Eco*RV site of pBluescript II SK+ (Stratagene). The fragment was then excised by digesting with *Xho*I and *Bam*HI, and inserted into pC7βG pre-digested with the same enzyme combination to remove the CMV promoter. Note that this doxycycline-responsive promoter is active in the absence of doxycycline.

A segment of the *CPB2* 3'-flanking region beginning at the stop codon and extending for 1 kb downstream was amplified from human genomic DNA. The segment was inserted into pC7βG digested with *Pac*I (just downstream of the translation termination cassette) and *Sal*I. In utilizing this downstream *Sal*I site in pC7βG, the SV40 polyA signal was removed prior to insertion of the genomic DNA segment. Our previous work showed that the SV40 polyA sequence suppresses the utilization of the native polyA signals in the *CPB2* 3'-UTR [18]. Therefore, in this new generation of fusion mRNA expression plasmids expressed transcripts that utilize these native polyA signals and generate three different polyadenylated species (data not shown). The TTP binding site in

the 3'-flanking region was mutated as previously described [21]. *CPB2* promoter constructs were described previously [6,18].

3.2.2 Mammalian cell culture and transfections

Cells were maintained in a humidified incubator at 37°C under a 95% room air/5% CO_2 atmosphere. HepG2 cells were cultured as previously described [21]. Inflammatory mediators were added, where indicated, at the following concentrations: TNFα (50 pg/mL), combination of IL-6 (10 ng/mL) and IL-1β (1 ng/mL), IL-8 (30 ng/mL), LPS (1 μg/mL), IL-10 (1 ng/mL) or IL-13 (10 ng/mL).The concentrations used for TNFα and IL-8 were as per manufacturer's suggestion for ED_{50}; the concentrations for IL-6 and IL-1β were obtained from ref.16, for LPS from ref.28, and for IL-10 and IL-13 from ref.45 The cells were incubated for up to 48 hours thereafter prior to isolation of conditioned media and total cellular lysates in lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) or extraction of RNA. Transfections of HepG2 cells were performed with Lipofectamine 2000 (Invitrogen) reagent as per the manufacturer's protocol, and in the presence of 100 ng/mL doxycycline (Clontech). After 48 hours, doxycycline was removed to induce transcription of the plasmid, the cells were seeded into 24-well plates and cultured for 16 hours. The next day, treatments were added, as indicated. After 24hrs of treatment, transcription was arrested by addition of doxycycline at 1 μg/mL, and RNA extracted at various time points thereafter. RNA isolation and real-time qRT-PCR were carried out as described previously [21].

3.2.3 TAFI activation and TAFIa assay

A highly sensitive functional assay for TAFIa was implemented as previously described [24]. Briefly, the assay is performed by mixing soluble fibrin degradation

products (FDPs) covalently attached to a quencher (QSY) with fluorescein-labeled plasminogen. The sample containing TAFIa is then added, and the rate of fluorescence increase due to removal of carboxyl-terminal lysine from FDPs and subsequent loss of plasminogen binding is measured with a fluorescence plate reader. The first step is quantitative activation of all TAFI present in the sample, which was carried out as described previously [25]. TAFIa concentrations measured by the TAFIa assay in each sample were divided by the respective total cellular protein values obtained using the BCA assay (Pierce Chemical) to account for the differences in cell numbers. Results were then normalized to the untreated medium sample for each time point, expressed as fraction per mg of total cellular protein.

3.2.4 RNA-binding protein purification and identification (RaPID)

The RaPID procedure relies on the use of two main constructs as previously described [26]. pN-RFPx24 and pUG34-MS2-CP-GFP-SBP, which were the kind gift from Dr. Jeffrey Gerst (Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel). Since pUG34-MS2-CP-GFP-SBP is a yeast plasmid, we first removed the sequence encoding the fusion protein CP-GFP-SBP by digestion with *Xba*I, and then inserted it into pcDNA-4B plasmid pre-digested with *Xba*I. The *CPB2* 3' flanking region was inserted into pN-RFPx24 plasmid by excising this segment from βG-*CPB2/*3'UTR (see above) with *Xho*I and *Sal*I , making the ends blunt with T4 DNA polymerase and inserting into pN-RFPx24 digested with *Bam*HI and blunted. HepG2 cells were transfected in 100-mm dishes with pcDNA4B-CP-GFP-SBP and pN-RFPx24-*CPB2*/3'UTR simultaneously using Lipofectamine 2000 reagent for 48 hours. Collection and lysis of cells was carried out as previously described [26], with a modification for cross-linking, which was done in phosphate-buffered saline (PBS) containing 0.5% (v/v)

formaldehyde for 10 min at 24°C with slow shaking. The pulldown assay was performed as previously described [26]. For western blot analyses, experiments were performed using SDS-PAGE on 4-15% polyacrylamide gradient gels under non-reducing conditions. The blots were incubated with HRP-linked polyclonal goat anti-human TTP or goat-anti human HuR antibody (Santa Cruz Biotechnology; both at 1:1000 dilution) in blocking buffer containing 3% (w/v) non-fat milk in 1× NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, 0.5% Triton X-100) and incubated at 4°C overnight. Immunoreactive bands were visualised using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and a FluorChem Q Gel Imaging System (Alpha Innotech).

3.2.5 RNA Immunoprecipitation

HepG2 cells were transfected in 100-mm dishes with βG-*CPB2/*3'UTR or βG-*CPB2/*3'UTRΔTTP plasmids for 48 hours, followed by crosslinking and lysis as described above for the RaPID procedure. Immunoprecipitation was carried out as described previously [27] using 1 mg of goat anti-human HuR antibody (Santa Cruz Biotechnology). All lysate samples were in parallel incubated with 1 mg of irrelevant antibody of the same isotype (goat-anti-donkey (Santa Cruz Biotechnology)). Mock IP reactions were also carried out, containing the lysis buffer and anti-HuR antibody. After the final wash, the supernatant was aspirated and 1 mL of TriZol (Invitrogen) was added to the beads. RNA was extracted, treated with DNase I for 1 hour and phenol/chloroform extracted. The amounts of β-globin fusion transcripts were quantified with real time qRT-PCR using primer and probe sets specific for rabbit β-globin and human GAPDH, as described above.

3.3 Results

3.3.1 Pro-inflammatory mediators decrease TAFI protein levels

In order to evaluate the effects of inflammatory mediators on the expression of TAFI in liver cells, we utilized a HepG2 (human hepatocellular carcinoma) cell model system – in which TAFI is expressed endogenously – and a recently-developed assay specific for TAFIa [24]. HepG2 cells were treated with various pro-inflammatory cytokines, namely TNFα, IL-6 in combination with IL-1β, and IL-8, as well as LPS which induces expression of pro-inflammatory cytokines in HepG2 cells [28]. Conditioned medium was collected at various time points for quantification of TAFI secretion using the TAFIa assay. After 24 hours, TAFI protein levels decreased by 60% in the presence of TNFα and LPS compared to the untreated control (Fig.3.1). The capacity of these mediators to decrease TAFI protein was maximal at this point, since the decrement remained at 60% after 48 hours of treatment. Combination treatment with IL-6 and IL1-β resulted in 45% decrease in TAFI levels at 24 hours compared to non-treated control, and culminated at 60% decrease in TAFI levels after 48 hours. Interestingly, treatment of cells with IL-8 caused only a modest decrease that was observable after 48 hours and which was not statistically significant.

We then hypothesized that treatments with anti-inflammatory cytokines may cause the opposite effects on TAFI levels. IL-10 and IL-13 have been recognized to down-regulate pro-inflammatory cytokine and chemokine production after the appropriate inflammatory response had occurred, in order to down-regulate inflammation. IL-13 did not significantly affect TAFI protein levels in HepG2 cells (data not shown), whereas the treatment of cells with IL-10 caused a 2-fold increase in TAFI protein levels after 48 hours (Fig.3.1).

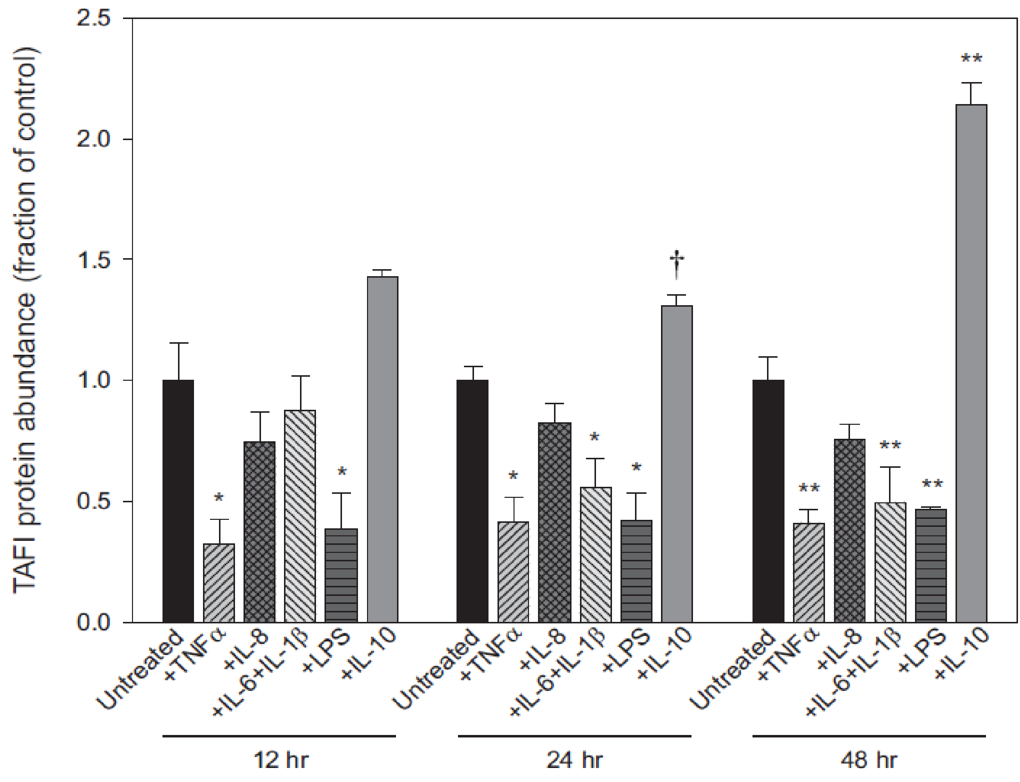

Figure 3.1 Inflammatory mediators modulate TAFI protein levels in HepG2 cells. HepG2 cells were grown in 6-well plates and treated with pro-inflammatory cytokines TNFα (50 pg/ml), IL-8 (30ng/ml) , IL-6 (10ng/ml) in combination with IL-1β (1ng/ml), LPS (1µg/ml) or the anti-inflammatory cytokine IL-10 (1ng/ml). Conditioned media was collected at various time points and TAFI was quantitatively activated with thrombin-thrombomodulin prior to a functional assay specific for TAFIa. TAFIa amounts in each sample are corrected to their corresponding total cellular protein content and expressed relative to the TAFIa amount present in the untreated samples at the respective time points. The data shown are the mean ±s.e.m.of three independent experiments. †: $p < 0.05$; *: $p < 0.01$; ** $p < 0.001$ versus untreated control by Student's t-test.

3.3.2 Effects of pro- and anti-inflammatory cytokines and mediators on *CPB2* mRNA abundance and promoter activity

The effects of inflammatory mediators on endogenous TAFI protein levels in HepG2 cells prompted us to investigate regulation of *CPB2* gene expression to determine the mechanisms underlying these effects. We first measured endogenous *CPB2* mRNA levels with real-time qRT-PCR following 24-hour incubation. Inflammatory cytokines modulate *CPB2* mRNA levels in a way that is reflective of TAFI protein levels for each treatment group (Fig. 3.2). *CPB2* mRNA abundance was reduced in cells treated with TNFα, IL-6/IL-1β, and LPS; it remained unchanged in the cells treated with IL-8 and was increased in IL-10 treated cells.

Next, we studied the effects of the inflammatory mediators on *CPB2* promoter activity using luciferase reporter gene constructs whose expression is under the control of the human *CPB2* 5'-flanking region. The constructs contain systematic 5'-deletions of genomic DNA to allow for the localization of any *cis*-acting elements. HepG2 cells were transiently transfected with the constructs, followed by the addition of inflammatory mediators. The treatments were maintained for 24 hours and luciferase activity was measured. None of the constructs appeared to be affected by either pro- or anti-inflammatory mediators (Fig. 3.3). As expected, the -73 and empty (pGL3 Basic) constructs showed vastly reduced luciferase activity. Therefore, the observed changes in mRNA levels are not attributable to changes in *CPB2* promoter activity.

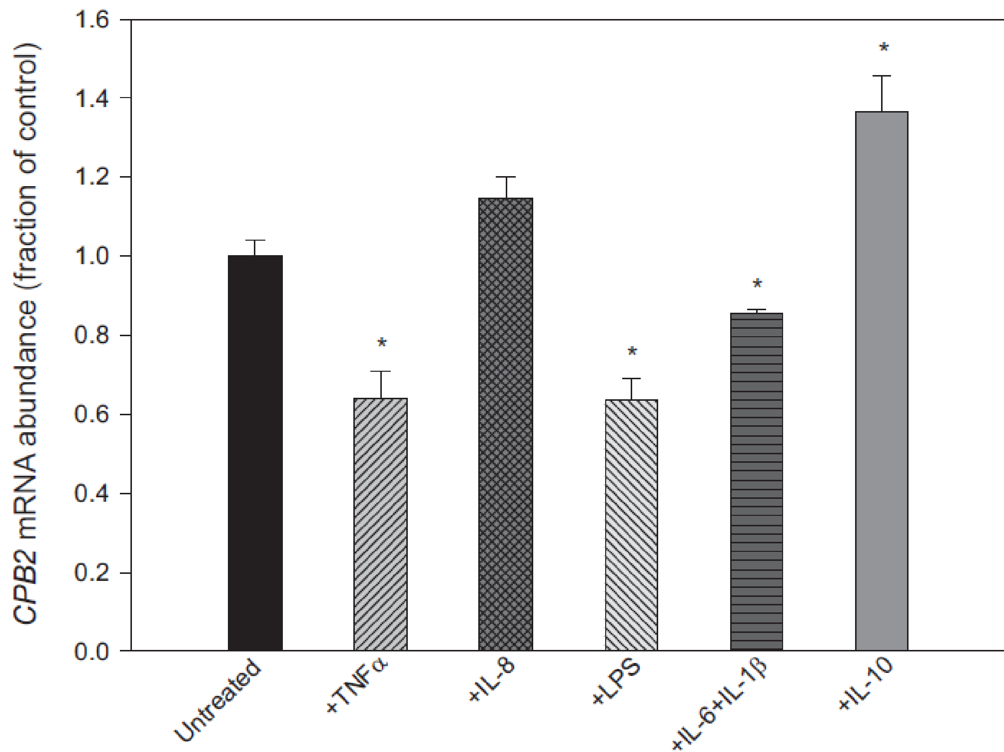

Figure 3.2 Inflammatory mediators modulate abundance of endogenous *CPB2* mRNA. HepG2 cells were treated with indicated inflammatory mediators for 24 hours, followed by isolation of total RNA and DNase I digestion. Endogenous *CPB2* mRNA was quantitated with real-time qRT-PCR using Taq-based chemistry in a multiplex reaction containing primer and probe sets specific for human TAFI and GAPDH. The relative abundance of TAFI mRNA for each sample was then normalized to untreated control. The data shown are the mean ± s.e.m.of three independent experiments.
*: $p < 0.01$ versus untreated control by Student's t-test.

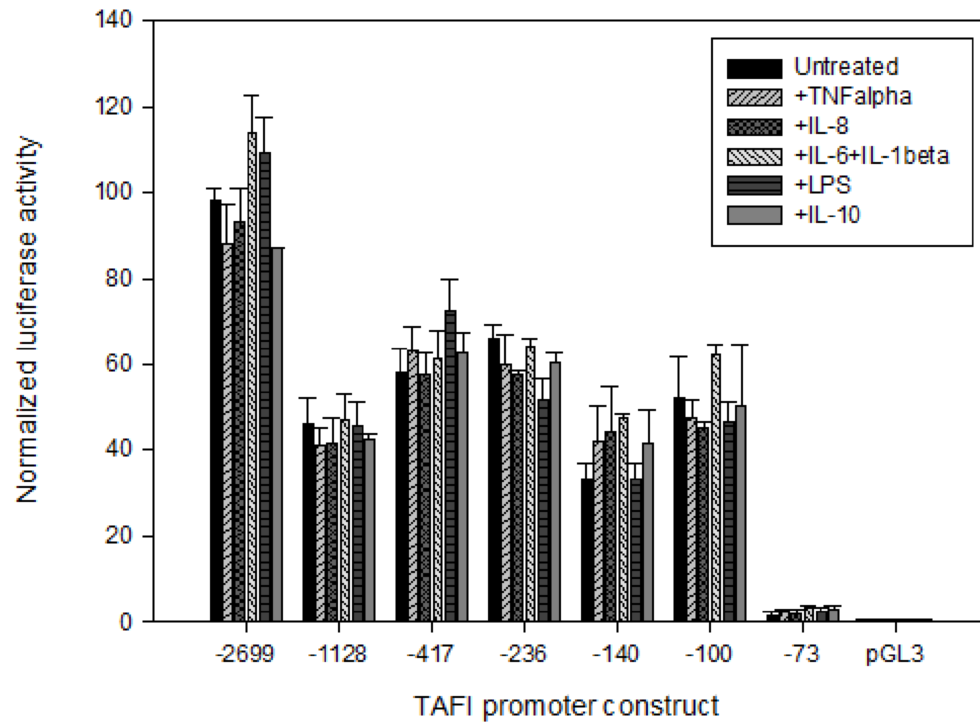

Figure 3.3 Inflammatory mediators do not influence transcription of the *CPB2* promoter. Firefly luciferase reporter plasmids containing systematic deletions of the *CPB2* 5'-flanking region were used to assess the effect of inflammatory mediators on *TAFI* promoter activity. HepG2 cells were transiently transfected with luciferase fusion constructs, followed by the addition of inflammatory mediators. The treatments were maintained for 24 hours and relative luciferase activity was measured, using *Renilla* luciferase as an internal control. Values are expressed relative to the -2699 construct in the absence of cytokine treatment. The data shown are the mean ± s.e.m. of three independent experiments.

3.3.4 Effects of pro- and anti-inflammatory cytokines and mediators on *CPB2* mRNA stability

We next sought to determine whether their regulatory effects are exerted through changes in mRNA stability mediated by the 3'-UTR. We used our β-globin reporter mRNA system for these studies, as previously reported [21], improved by several modifications. The βG-*CPB2*/3'-UTR plasmid utilized in these experiments contains a 460 bp sequence of rabbit β-globin cDNA whose expression in this system is driven by a doxycycline-responsive promoter. We conducted the experiments in HepG2 cells that stably express the transactivator protein necessary for the activity of this promoter. Upon addition of doxycycline, the binding of the transactivator protein is impeded, resulting in transcriptional shut-off, allowing for the subsequent measurement of mRNA half-life. This system allows us to selectively inhibit the transcription of our constructs only, without affecting the cellular transcription machinery, a consequence that is encountered when other drugs are used to stop transcription (i.e. actinomycin D). We then cloned a 1-kb segment of the *CPB2* 3'-flanking region downstream of the β-globin cDNA sequence, encompassing the native polyA signals and the surrounding regulatory elements (approximately 450 bp downstream of the 3'-most polyA site). By including these elements and deleting the SV40 polyA signal present in the original pC7βG plasmid, utilization of binding sites on fusion transcripts by the various *trans*-acting factors is expected to be reflective of what occurs on the endogenous *CPB2* counterparts.

HepG2 cells were treated with the inflammatory mediators under study for 24 hours, and doxycycline was added in the absence of the treatments. The cell lysates were collected at various time points thereafter, up to 8 hours. RNA was extracted and subjected to multiplex real-time qRT-PCR analysis, using primers and probes specific for

rabbit β-globin and GAPDH, which was used for normalization. Linear regression analysis was performed and fusion transcript mRNA half-lives were determined (Table 1). Treatments with TNFα, LPS and IL-6 together with IL-1β resulted in 30% destabilization of the fusion mRNA, while IL-8 treatment did not cause significant change in mRNA half-life (Table 3.1; Fig. 3.4A). Interestingly, treatment of cells with IL-10 produced a stabilizing effect, resulting in a 37% increase in fusion mRNA half-life (Table 3.1; Fig. 3.4A). These results are in line with the observations of TAFI protein secretion and *CPB2* mRNA abundance, and suggest that the observed effects of the anti- and pro-inflammatory mediators and cytokines are, at least in part, attributable to changes in mRNA stability.

Our most recent work demonstrated that TTP is capable of binding to the *CPB2* 3'-UTR, exerting destabilizing effects [21]. TTP has been shown to cause similar effects on mRNAs encoding many cytokines, transcription factors, and oncogenes [2]. Moreover, binding of TTP to the TNFα 3'-UTR is modulated during inflammatory response [29]. Initially, in order to allow rapid synthesis and production, the binding of TTP to the TNFα 3'-UTR is decreased. After the proper inflammatory response has ensued, TTP rapidly targets TNFα mRNA for degradation, to prevent exaggerated inflammation and delays in its downregulation. To assess the potential role of TTP in mediating the destabilizing effects of the pro-inflammatory mediators we observed on the fusion mRNA containing *CPB2* 3'-UTR, we introduced mutations in the TTP binding site within the *CPB2* 3'-flanking region in the context of our β-globin fusion mRNA constructs (βG-*CPB2*/3'-UTRΔTTP). These mutations abolish TTP binding and stabilize the fusion transcripts by 67% [21]. Treatments of cells expressing the fusion mRNAs containing the TTP mutation with pro-inflammatory mediators under study did not result in significant changes in half-

lives compared to the untreated control (Table 3.1; Fig. 3.4B). However, treatment of cells expressing the mutant fusion transcript with IL-10 resulted in further stabilization of the mRNAs (56% increase in half-life). Therefore, modulation of *CPB2* mRNA stability in the presence of pro-inflammatory mediators is mediated by TTP, while modulation by the anti-inflammatory IL-10 is not.

3.3.5 Identification of HuR as a trans-acting protein binding to the *CPB2* 3'-UTR

Our previous report describes the presence of one stability element upstream of the first polyA site within the *CPB2* 3'-UTR, followed by two instability elements upstream of the second and third polyA sites, respectively [21]. We identified TTP as the *trans*-acting factor binding to the 3'-most instability element. Here we set out to identify other ARE-binding proteins (ABPs) acting on these *cis*-elements. To conduct this analysis, we adapted a recently described aptamer-based mRNA affinity purification technique for the identification of RNA and protein factors present in ribonucleoprotein complexes, called RNA purification and identification (RaPID) [26]. This method exploits the high affinity interaction between MS2 aptamer sequences and bacteriophage coat- binding protein, as well as high affinity interaction between streptavidin and streptavidin-binding protein for the affinity chromatography purification step. We inserted the 1 kb segment of the *CPB2* 3'-flanking region downstream of the 24 repeats of MS2 aptamer sequences in one construct. Therefore, any protein factors bound to the 3'-UTR will be captured with this transcript (bait construct). The second construct encodes a fusion protein consisting of bacteriophage coat protein for high affinity binding to the MS2 aptamers on one end, and streptavidin-binding protein on the other end for binding to streptavidin-coated beads, with green fluorescence protein in the middle of the fusion protein. HepG2 cells were co-transfected with both plasmids and lysates were subjected

to affinity chromatography. The associated proteins were eluted off the beads; to verify occupancy of the 3'-UTR by TTP, the eluate was subjected to Western blot analysis with an anti-TTP antibody. Immunoreactive bands specific for TTP were only present in the lane containing lysate from cells transfected with the *CPB2*/3'-UTR-containing bait plasmid, and not in the lane corresponding to the 3'UTR-less bait (Fig. 3.5A).

An intriguing candidate for a trans-acting factor binding to the *CPB2* 3'-UTR is HuR. Bioinformatic analysis of the sequence upstream of the first polyA site revealed its U-richness (a requirement for HuR binding) and this region also contains a *cis*-acting stability element [21]. We therefore performed the RaPID experiment, followed by immunoblotting with an anti-HuR antibody. The immunoreactive band in the lane corresponding to the sample containing the *CPB2* 3'-UTR indicates that HuR is indeed capable of binding (Fig. 3.5B).

3.3.6 Effects of pro- and anti-inflammatory cytokines and mediators on HuR binding to the *CPB2* 3'-UTR

TTP and HuR exert opposing effects on target transcript stability. Moreover, it has been shown that the binding may be competitive (i.e. mutually exclusive) [30]. In fact, regulation of the chief inflammatory cytokine TNFα has been shown to be controlled by the p38/MAPK-activated protein kinases; MK2-driven exchange between TTP and HuR at the TNFα 3'-UTR in macrophages allows the development of prompt inflammatory response, followed by appropriate downregulation [30]. We sought to investigate whether the same paradigm extends to the regulation of *CPB2* mRNA in hepatic cells.

HepG2 cells were transfected with β-globin fusion constructs and treated with the same schedule of pro- and anti-inflammatory mediators as described above. Cellular lysates were then subjected to immunoprecipitation using antibody specific for HuR. The

immunoprecipitates were extensively washed and the associated transcripts were eluted off the beads with TriZol reagent. RNA was extracted, treated with DNase I, and subjected to real-time qRT- PCR using a primer and probe set specific for rabbit β-globin cDNA. In parallel, control immunoprecipitation reactions were carried out for each treatment sample using an irrelevant antibody of the same isotype. Immunoprecipitates from cells treated with pro-inflammatory mediators were depleted of the βG-fusion mRNA compared to the untreated sample (Fig. 3.6A), indicating reduced occupancy of the *CPB2* 3'-UTR by HuR. Conversely, immunoprecipitates from cells treated with the anti-inflammatory cytokine IL-10 were significantly enriched with the βG-fusion transcript (Fig. 6A).

Next we examined whether HuR and TTP may share the binding site within the *CPB2* 3'-UTR by performing immunoprecipitation experiments using our ΔTTP mutant β-globin fusion constructs. TTP and HuR appear to contact discrete sites within *CPB2* 3'-UTR as there was no difference in the occupancy of HuR between the wild-type and ΔTTP mutant fusion mRNA's (Fig. 3.6B, inset). However, cytokine treatment of cells transfected with the ΔTTP mutant plasmid increased occupancy of the fusion transcripts with HuR, with the exception of IL-8 (Fig. 3.6B), indicating that the absence of TTP bound to the transcript promotes HuR binding in the presence of both pro- and anti-inflammatory stimuli. This may suggest that the binding of TTP and HuR are mutually exclusive despite the recognition of distinct sites on the *CPB2* 3'-UTR.

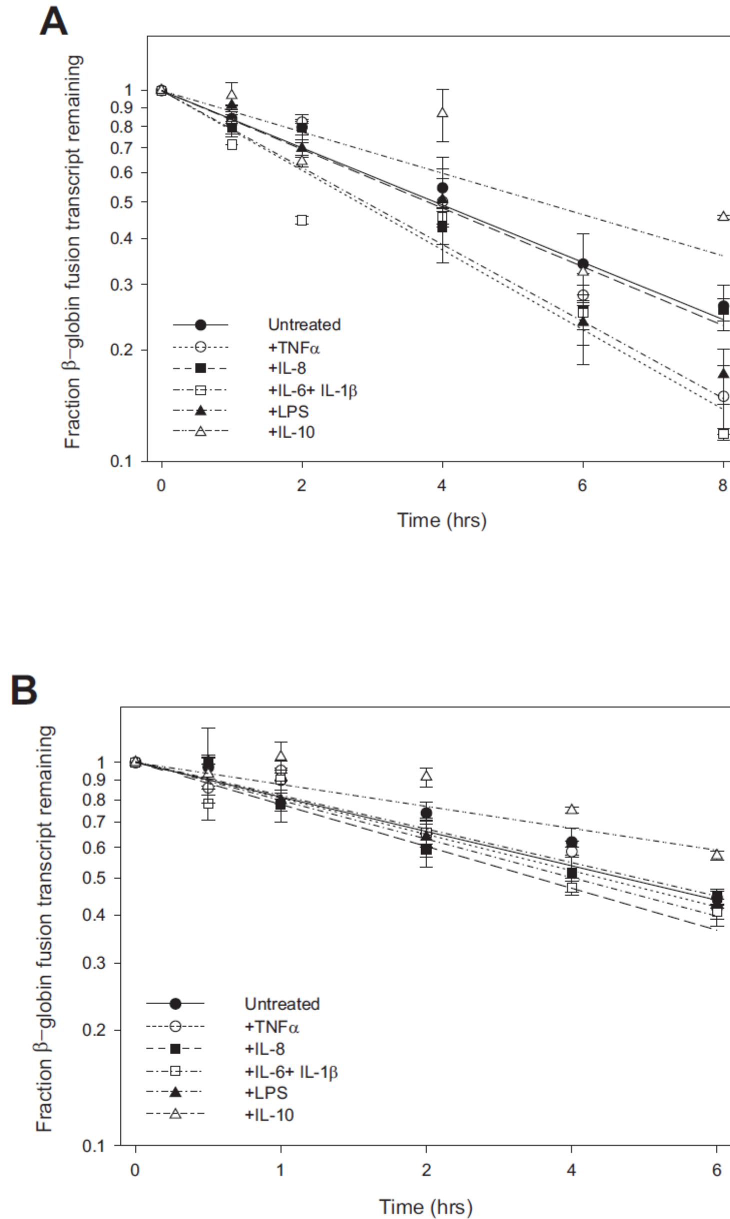

Figure 3.4 ***CPB2*** **mRNA stability is modulated by inflammatory mediators.** The effects of inflammatory mediators on mRNA stability were assessed using our β-globin fusion constructs whose expression is under the control of doxycycline-repressible promoter, containing 1kb of the *CPB2* 3'-flanking region, either with intact TTP binding site (β-globin-*CPB2* 3'-UTR) (Panel A) or with TTP binding site mutation (β-globin-ΔTTP 3'-UTR) (Panel B). Transfected HepG2 cells were treated with inflammatory mediators for 24 hours, and doxycycline was added to the final concentration of 1μg/ml in the absence of the treatments. The cell lysates were collected at various time points thereafter, up to 8 hours. RNA was extracted and subjected to multiplex real-time qRT- PCR analysis, using primer and probe sets specific for rabbit β-globin and human GAPDH. Linear regression analysis was performed and fusion transcript half-lives were determined (Table 3.1). The data shown are the mean ± s.e.m. of three independent experiments.

Table 3.1 Stabilities of fusion mRNAs after cytokine treatment

Treatment	β-globin-TAFI3'-UTR t(1/2) (hrs)	β-globin-ΔTTP 3'-UTR t(1/2) (hrs)
Untreated	3.9 ± 0.4	6.7 ± 0.5
+TNFα	2.8 ± 0.1*	6.4 ± 0.8
+IL-8	3.8 ± 0.2	5.5 ± 0.1*
+IL-6/IL-1β	2.9 ± 0.2*	6.0 ± 0.4
+LPS	2.9 ± 0.1*	6.9 ± 0.8
+IL-10	5.4 ± 0.1*	10.5 ± 0.5*

*: $p < 0.01$ versus untreated control by Student's t-test

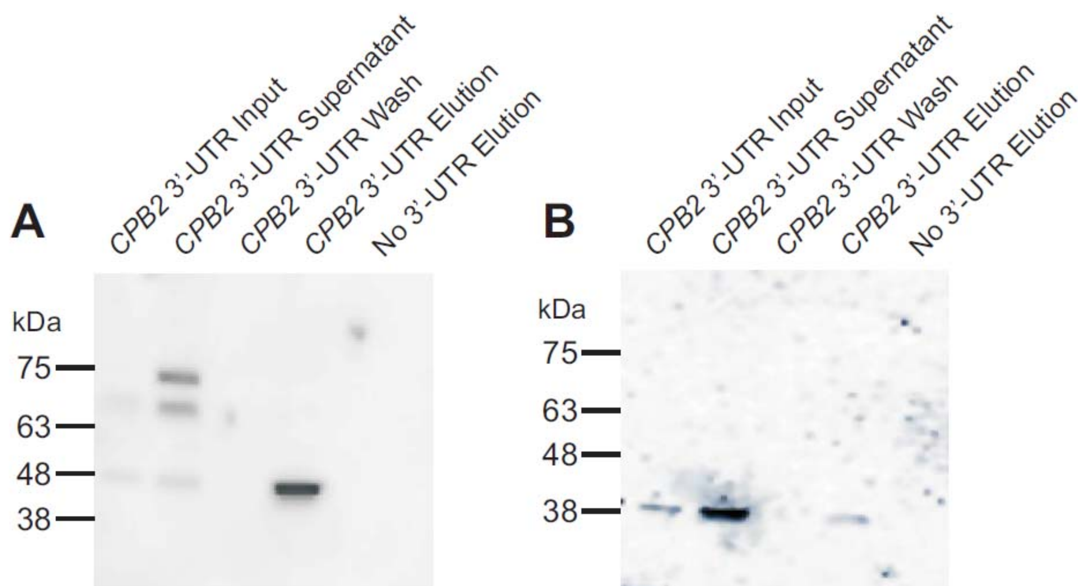

Figure 3.5 Identification of *trans*-acting factors binding to the *CPB2* 3'-UTR. We adapted an aptamer-based mRNA affinity purification technique for the identification of RNA and protein factors present in ribonucleoprotein complexes. HepG2 cells were co-transfected with the 'bait' construct encoding the aptamer repeats and the *CPB2* 3'-UTR and the 'hunter' construct encoding the aptamer-binding protein and lysates were subjected to affinity chromatography. The input, supernatant, wash and elution fractions from the chromatography were subjected to Western blot analysis using an anti-TTP antibody (Panel A) or anti-HuR antibody (Panel B). Control experiments were performed using a 'bait' construct lacking the *CPB2* 3'-UTR and the elution fraction was also included on the Western blots (No 3'-UTR).

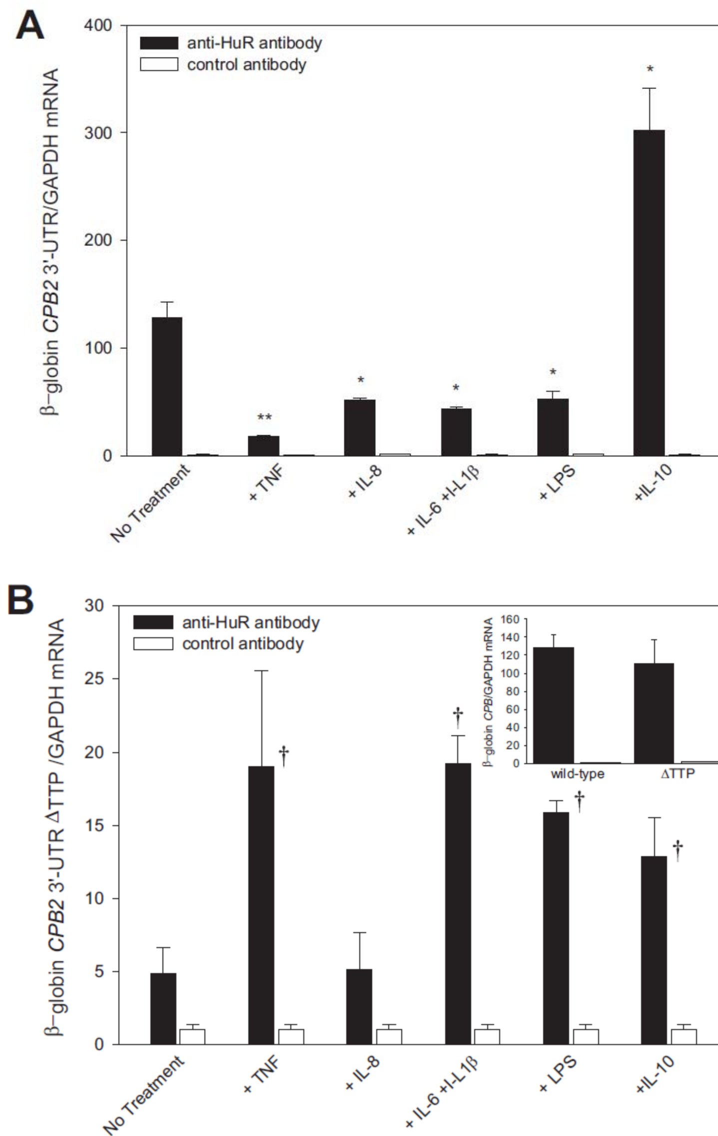

Figure 3.6 Inflammatory mediators modulate HuR binding to the *CPB2* 3'-UTR. HepG2 cells were transfected with plasmids encoding β-globin- *CPB2* 3'-UTR fusion mRNA (Panel A) or β-globin-ΔTTP 3'-UTR fusion mRNA (Panel B), and treated for 24 hours with the indicated inflammatory mediators. RNA immunoprecipitation experiments were carried using anti-HuR antibody and protein A agarose beads. RNA was extracted from immunoprecipitates with TriZol, and quantitated with real-time qRT-PCR. Parallel reactions were carried out using non-specific antibody of the same isotype (control). Inset: Direct comparison of the qRT-PCR signal from the respective fusion transcripts after immunopreciptation The data shown are the mean ± s.e.m. of three independent experiments. †: $p < 0.05$; *: $p < 0.01$; ** $p < 0.001$ versus untreated control by Student's t-test.

3.4 Discussion

Plasma TAFI concentrations vary considerably in the human population, with the most recent estimate ranging from 50% to greater than 200% of the mean value [31]. The majority of this variation appears to be attributable to gene regulatory events, since traditional risk factors for cardiovascular disease were shown to have little impact on TAFI levels and genetic factors account for about a quarter of the variation [32]. Many hormonal factors and disease states have been shown to affect TAFI concentrations [33, 34], including pregnancy; pre-eclampsia; use of oral contraceptive or hormone replacement therapy in women; renal disease; multiple myeloma; thyroid status, and various forms of inflammatory conditions such as Behçet's disease, inflammatory bowel disease; sepsis; and complications of sepsis including disseminated intravascular coagulation (DIC) and multiple organ dysfunction syndrome (MODS). In the present study we set out to gain further insights into the transcriptional and post-transcriptional regulatory mechanisms governing TAFI expression, focusing on the role of inflammatory mediators. Changes in plasma TAFI concentrations in the setting of inflammation may alter the host defense response in terms of both hemostasis/fibrinolysis and inflammation itself, as TAFI has been shown to influence both of these pathways.

We investigated the effects of pro-inflammatory cytokines and modulators on TAFI protein levels in HepG2 cells using a sensitive and quantitative functional assay for TAFIa. TNFα, IL-6 plus IL-1β and LPS decreased TAFI protein levels in conditioned medium of HepG2 cells, while IL-8 had no significant effect. Interestingly, treatment of HepG2 cells with the anti-inflammatory cytokine IL-10 resulted in increased TAFI protein levels, whereas IL-13 had no effect.

Since our initial report on the role of *CPB2* 3'-UTR in dictating mRNA stability and the effect of IL-6 and IL-1β on both abundance and mRNA stability [16,18], we have continued to investigate the mechanistic basis of these observations. Here we identified a regulatory role of TTP in modulating *CPB2* mRNA stability in the presence of inflammatory cytokines and mediators. TTP binds to mRNAs and destabilizes them by promoting their deadenylation and subsequent degradation [2,29]. TTP is present in the liver, and certainly in hepatocytes, as evidenced by the presence in HepG2 cells, and its expression is induced by TNFα and bacterial LPS. Therefore, TTP is a plausible candidate for mediating both constitutive and regulated *CPB2* mRNA stability in HepG2 cells.

To examine the role of TTP in these events, we selectively mutated the TTP binding site in *CPB2* 3'UTR and studied the effects on mRNA stability. To do this, we constructed "second generation" β-globin fusion plasmids in which the strong SV40 polyA signal was removed from the plasmid backbone and a total of 1 kb of 3'-flanking sequence from the *CPB2* gene inserted. Using these constructs we would expect that the stability and polyadenylation of the resultant fusion transcript would faithfully recapitulate that seen in the endogenous *CPB2* mRNA in HepG2 cells; preliminary studies indicate that this is the case (data not shown). In addition, we replaced the cytomegalovirus promoter in the original plasmid with a doxycycline-repressible promoter to allow selective transcriptional shut-off of the fusion constructs only, circumventing the need for other transcriptional inhibitor drugs, such as actinomycin D, which affects global transcription, including that of genes encoding short-lived mRNA species whose protein products have potentially important regulatory roles. The ability of these inflammatory mediators to modulate endogenous *CPB2* mRNA abundance as

shown in Fig. 2, prompted us to investigate whether the modulation occurs at the transcriptional or post-transcriptional level, or both. Using our luciferase *CPB2* promoter constructs we detected no difference in promoter activity in the presence of inflammatory mediators under study (Fig. 3.3). A recent report showed that a hypolipidaemic drug, PPARα agonist WY14643, mediated reduction of TAFI protein levels in HepG2 cells caused by a decrease in *CPB2* mRNA stability, without affecting transcriptional aspect of *CPB2* gene expression [35]. Here, we uncovered that inflammatory mediators utilize a similar mechanism to reduce TAFI protein expression through exclusively post-transcriptional effects, although they classically operate through transcriptional mechanisms, via induction of NFκB, its translocation into the nucleus and subsequent activation of transcription of genes encoding acute phase proteins [36]. In fact, we recently reported transcriptional activation of the mouse *CPB2* gene following TNFα treatment of mouse hepatocytes and demonstrated that this effect is indeed NFκB-dependent [37]. However, this report also demonstrated that the consensus NFκB binding site is absent from the human *CPB2* promoter, which may explain the reason behind post-transcriptional modulation of *CPB2* mRNA stability as the chief operating mechanism in human hepatocytes.

The present work indicates that the stability of the β-globin fusion transcripts in HepG2 cells was reduced by one third compared to untreated control cells, when TNFα, IL-6 plus IL-1β or LPS treatments were administered (Table 3.1; Fig. 3.4A). IL-8 did not alter the fusion mRNA stability, consistent with the observations on TAFI protein and endogenous *CPB2* mRNA levels in the presence of this cytokine. In our laboratory we have observed that IL-8 plays a role in modulating the levels of TAFI protein produced by THP-1 macrophages (unpublished data). In fact, IL-8 signaling is the chief pathway

operating during vascular inflammation, and in light of our observation on macrophage-secreted TAFI, may be involved in modulation of extra-hepatic pool of TAFI in the vasculature, which may have pleiotropic effects on thrombus formation and lysis, leukocyte adhesion and plaque formation, all of which are contributing events to atherosclerosis. The destabilization of the β-globin fusion transcripts by pro-inflammatory cytokines was abolished by mutations in the TTP binding site (Table 3.1; Fig. 3.4B). These results suggest that TNFα, IL-6 in combination with IL-1β, and LPS-induced down-regulation of TAFI protein and mRNA levels is mediated through TTP-induced destabilization. The IL-10-mediated increase in TAFI protein levels is clearly attributable to the increase in mRNA abundance (Fig. 3.2A) due to increased mRNA stability (Fig. 3.3A). However, TTP does not appear to play a role in this event, as the mutant fusion mRNA was equally stabilized upon IL-10 treatment (Table 3.1; Fig. 3.4B).

In addition to TTP, another ABP that is a candidate to contact *CPB2 cis*-sequences within the 3'-UTR is HuR. We adapted the RaPID technique to isolate proteins associated with the *CPB2* 3-'UTR using affinity chromatography, and the presence of the immunoreactive band in the lane corresponding to the elution step (Fig. 3.5B) confirms the association between HuR and the *CPB2* 3'-UTR. Transcripts of many cytokines destabilized by TTP, such as TNFα, IL-8 and COX-2, have been shown to possess a binding site for HuR which acts to stabilize them [38-40]. These two ABPs play opposing roles and together dictate the abundance of target transcript. In fact, TTP and HuR have also been shown to bind to the transcripts encoding themselves as well as each other, and these events are coordinated by microRNAs [41]. Indeed, HuR is often referred to as the "regulator of regulators', much like TNFα is often referred to as the master cytokine in inflammation [30]. In the current report, we investigated whether the reduced *CPB2*

mRNA stability in the presence of pro-inflammatory mediators is also accompanied by a decrease in HuR binding to the 3'-UTR. RNA-immunoprecipitation experiments were performed with anti-HuR antibody and the amount of associated β-globin fusion transcript was decreased in immunoprecipitates from cells treated with pro-inflammatory cytokines, while it increased in immunoprecipitates from cells treated with IL-10, strongly supporting the role for both TTP and HuR in dictating *CPB2* mRNA abundance, exerting opposite effects. It is easy to envision that TTP and HuR, as functional competitors in determining *CPB2* mRNA stability, may be sharing the binding site and that the binding of one impedes the binding of the other. However, RNA-IP experiments with the HuR antibody showed that the absence of TTP binding site did not alter the binding of HuR (Fig. 3.6B, inset). Therefore, TTP and HuR contact discrete sites within *CPB2* 3'-UTR.

It has been also been reported that in situations where even if TTP and HuR bind discrete sites, the binding of one excludes the binding of the other, possibly through mRNA secondary structure perturbations or steric hindrance [40,42]. In fact, immunoprecipitates from cells expressing β-globin-ΔTTP 3'-UTR (which cannot bind TTP) in the presence of inflammatory mediators were enriched in the fusion mRNAs compared to untreated control, indicating that binding of TTP and HuR may be mutually exclusive in the presence of inflammatory cytokines, despite their preference for different binding sites. Therefore, the decrease in TAFI protein levels upon treatment of HepG2 cells with TNFα, or IL6 plus IL1β, or LPS can be attributed to decreased mRNA stability mediated by TTP with decreased binding of HuR, and thus decreased availability of translatable transcripts.

Anti-inflammatory cytokine IL-10 caused an increase of TAFI protein levels in conditioned medium of HepG2 cells by two-fold, owing to an increase in mRNA stability. Although the mechanisms underlying these effects of IL-10 remain unknown, they do not appear to be TTP-mediated. Perhaps the anti-inflammatory treatment employs mechanisms that act through the microRNA pathway, as was recently demonstrated for miR-155 in murine macrophages [43]. MiR-155 enhances the expression of pro-inflammatory cytokines, such as TNFα, and suppresses expression of anti-inflammatory molecules, such as SOCS1. Interleukin-10 treatment did not affect the transcription of the miR-155 host gene nor the nuclear export of pre-miR-155, but rather destabilized both pri-miR-155 and pre-miR-155 transcripts, as well as interfered with the final maturation of miR-155. Therefore, the orchestration of factors involved in the post-transcriptional regulation of the *CPB2* mRNA, both *cis*- and *trans*-, appears to be multifaceted and this report supports the roles of TTP and HuR in this process.

In conclusion, certain pro-inflammatory mediators decrease TAFI protein levels through mechanisms acting at the level of post-transcriptional regulation. TTP appears to be the master mediator of these effects in hepatocytes. Additionally, this report is to our knowledge the first to identify HuR as ABP acting on *CPB2* 3'-UTR, which may contribute to the modulation of stability of the *CPB2* transcripts in an inflammatory environment. The cytokine involved in coordinating anti-inflammatory processes, IL-10, increased TAFI levels in HepG2 cells, and this effect can be attributed to increased stability of the transcript. Deregulated balance between TTP and HuR observed in cancer [42], endothelial dysfunction and vascular inflammation [44], may represent at least one of the underlying mechanisms for variation in plasma TAFI concentrations observed in patients with these pathologies.

3.5 References:

1. Anderson P. *Post-transcriptional control of cytokine production.* Nat Immunol 2009; 9:p. 353-359.
2. Carrick DM, Lai WS, Blackshear PJ. *The tandem CCCH zinc finger protein tristetraprolin and its relevance to cytokine mRNA turnover and arthritis*. Arthritis Res Ther 2004; 6: p.248-264.
3. Srikantan S, Gorospe M. *HuR function in disease*. Front Biosci 2012; 17: p.189-205.
4. Foley JH, Kim PY, Mutch NJ, Gils A. *Insights into thrombin activatable fibrinolysis inhibitor function and regulation*. J Thromb Haemost 2013; **11Suppl 1**:p. 306-315.
5. Leung LL, Nishimura T, Myles T. *Regulation of tissue inflammation by thrombin-activatable carboxypeptidase B (or TAFI*). Adv Exp Med Biol 2008; 632: p.61-69.
6. Boffa MB, Reid TS, Joo E, et al. *Characterization of the gene encoding human TAFI (thrombin-activable fibrinolysis inhibitor; plasma procarboxypeptidase B*. Biochemistry 1999; 38: p.6547-6558.
7. Van Thiel DH, George M, Fareed J. *Low levels of thrombin activatable fibrinolysis inhibitor (TAFI) in patients with chronic liver disease.* Thromb Haemost 2001; 85: p.667-670.
8. Bajzar L, Manuel R, Nesheim ME. *Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor.* J Biol Chem 1995; 270: p.14477-14484.
9. Mao SS, Cooper CM, Wood T, et al. *Characterization of plasmin-mediated activation of plasma procarboxypeptidase B.* Modulation by glycosaminoglycans. J Biol Chem 1999; p.274: 35046-35052.
10. Bajzar L, Morser J, Nesheim M. *TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex*. J Biol Chem 1996; 271: p.16603-16608.
11. Redlitz A, Tan AK, Eaton DL, Plow EF. *Plasma carboxypeptidases as regulators of the plasminogen system.* J Clin Invest 1995; 96:p. 2534-2538.
12. Campbell WD, Lazoura E, Okada N, Okada H. *Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N*. Microbiol Immunol 2002; 6:p. 131-134.
13. Hendriks D, Wang W, van Sande M, Scharpé S. *Human serum carboxypeptidase U: a new kininase?* Agents Actions Suppl 1992; 38:p.407-413.
14. Myles T, Nishimura T, Yun TH, et al. *Thrombin activatable fibrinolysis inhibitor, a potential regulator of vascular inflammation.* J Biol Chem 2003; 278: p.51059-51067.
15. Du XY, Zabel BA, Myles T, et al. *Regulation of chemerin bioactivity by plasma carboxypeptidase N, carboxypeptidase B (activated thrombin-activable fibrinolysis inhibitor), and platelets.* J Biol Chem 2009; 284: p.751-758.
16. Boffa MB, Hamill JD, Maret D, et al. *Acute phase mediators modulate thrombin-activable fibrinolysis inhibitor (TAFI) gene expression in HepG2 cells*. J Biol Chem 2003; 278: p.9250-9257.

17. Lin JH, Garand M, Zagorac B, et al. *Identification of human thrombin-activatable fibrinolysis inhibitor in vascular and inflammatory cells.* Thromb Haemost 2011; 105: p.999-1009.
18. Maret D, Boffa MB, Brien DF, et al. *Role of mRNA transcript stability in modulation of expression of the gene encoding thrombin activable fibrinolysis inhibitor.* J Thromb Haemost 2004; 2:p. 1969-1979.
19. Garand M, Bastajian N, Nesheim ME, et al. *Molecular analysis of the human thrombin-activatable fibrinolysis inhibitor gene promoter*. Br J Haematol 2007; 138:p. 231-244.
20. Boffa MB, Maret D, Hamill JD, et al. *Effect of single nucleotide polymorphisms on expression of the gene encoding thrombin-activatable fibrinolysis inhibitor: a functional analysis*. Blood 2008; 111: p.183-189.
21. Novakovic D, Kuo AC, Lin JH, et al. *Identification of tristetraprolin as a factor that modulates the stability of the TAFI transcript through binding to the 3'-untranslated region.* J Thromb Haemost 2012; 10:p. 887-894.
22. Garand M, Lin JH, Zagorac B, et al. *Regulation of the gene encoding human thrombin-activatable fibrinolysis inhibitor by estrogen and progesterone*. Blood Coagul Fibrinolysis 2013; 24: p.393-404.
23. Peng SS, Chen CY, Shyu AB. *Functional characterisation of a non-AUUUA AU-rich element from the c-jun proto-oncogene mRNA: evidence for a novel class of AU-rich elements*. Mol Cell Biol 1996; 16: p.1490-1499.
24. Kim PY, Foley J, Hsu G, et al. *An assay for measuring functional activated thrombin-activatable fibrinolysis inhibitor in plasma.* Anal Biochem 2008; 372: p.32-40.
25. Lin JH, Novakovic D, Rizzo CM, et al. *The mRNA encoding TAFI is alternatively spliced in different cell types and produces intracellular forms of the protein lacking TAFIa activity.* Thromb Haemost 2013; 109:p. 1033-1044.
26. Galgano A, Forrer M, Jaskiewicz L, et al. *Comparative analysis of mRNA targets for human PUF-family proteins suggests extensive interaction with the miRNA regulatory system.* PLoS One 2008; 3: e3164.
27. Slobodin B, Gerst JE. *A novel mRNA affinity purification technique for the identification of interacting proteins and transcripts in ribonucleoprotein complexes.* RNA 2010; 16:p. 2277-2290.
28. Gutiérrez-Ruiz MC, Quiroz SC, Souza V, et al. *Cytokines, growth factors, and oxidative stress in HepG2 cells treated with ethanol, acetaldehyde, and LPS.* Toxicology 1999; 134: p.197-207.
29. Hitti E, Iakovleva T, Brook M, et al. *Mitogen-activated protein kinase-activated protein kinase 2 regulates tumor necrosis factor mRNA stability and translation mainly by altering tristetraprolin expression, stability, and binding to adenine/uridine-rich element*. Mol Cell Biol 2006; 26: p.2399-2407.
30. Tiedje C, Ronkina N, Tehrani M, et al. *The p38/MK2-driven exchange between tristetraprolin and HuR regulates AU–rich element–dependent translation*. PLoS Genet 2012; 8: e1002977.
31. Frère C, Morange PE, Saut N, et al. *Quantification of thrombin activatable fibrinolysis inhibitor (TAFI) gene polymorphism effects on plasma levels of TAFI measured with assays insensitive to isoform-dependent artefact*. Thromb Haemost 2005; 94: p.373-379.

32. Frère C, Tregouet DA, Morange PE, et al. *Fine mapping of quantitative trait nucleotides underlying thrombin-activatable fibrinolysis inhibitor antigen levels by transethnic study*. Blood 2006; 108: p.1562-1568.
33. Juhan-Vague I, Renucci JF, Grimaux M, et al. *Thrombin-activatable fibrinolysis inhibitor antigen levels and cardiovascular risk factors*. Arterioscler Thromb Vasc Biol 2000; 20: p.2156-2161.
34. Boffa MB, Koschinsky ML. *Curiouser and curiouser: Recent advances in measurement of thrombin-activatable fibrinolysis inhibitor (TAFI) and in understanding its molecular genetics, gene regulation, and biological roles.* Clin Biochem 2007; 40: p.431-442.
35. Masuda Y , Saotome D, Takada K, et al. *Peroxisome proliferator-activated receptor-alpha agonists repress expression of thrombin activable fibrinolysis inhibitor by decreasing transcript stability*. Thromb Haemost 2012; 108: p.74-85.
36. Uhlar CM, Whitehead AS. *Serum Amyloid A, the major vertebrate acute-phase reactant.* Eur J Biochem 1999; 265:p.501-23.
37. Garand M, Lin JHH, Hill CE, Zagorac B, Koschinsky ML, Boffa MB. *Regulation of the mouse gene encoding TAFI by TNFα:role of NFκB binding site.* Cytokine 2012; 57:p.380-97.
38. Lebedeva S, Jens M, Theil K, et al. *Transcriptome-wide analysis of regulatory interactions of the RNA-binding protein HuR.* Mol Cell 2011; 43:p. 340-352.
39. López de Silanes I, Zhan M, et al. *Identification of a target RNA motif for RNA-binding protein HuR*. Proc Natl Acad Sci USA 2004; 101:p.2987-2992.
40. Srikantan S, Tominaga K, Gorospe M. *Functional interplay between RNA-binding protein HuR and microRNAs*. Curr Protein Pept Sci 2012; 13:p. 372-379.
41. Al-Ahmadi W, Al-Ghamdi M, Al-Souhibani N, Khabar KS. *miR-29a inhibition normalizes HuR over-expression and aberrant AU-rich mRNA stability in invasive cancer.* J Pathol 2013; 230: p.28-38.
42. Meisner NC, Hackermuller J, Uhl V, et al. *mRNA openers and closers: modulating AU-rich element-controlled mRNA stability by a molecular switch in mRNA secondary structure.* Chembiochem 2004; 5: p.1432-1447.
43. Cheung ST, So EY, Chang D, Ming-Lum A, Mui ALF. *Interleukin-10 inhibits Lipopolysaccharide induced miR-155 precursor stability and maturation.* PLoS One, 2013; 8:e71336.
44. Cheng HS, Sivachandran N, Lau A, Boudreau E, Zhao JL, Baltimore D, Delgado-Olguin P, Cybulsky MI, Fish JE. *MicroRNA-146 represses endothelial activation by inhibiting pro-inflammatory pathways.* EMBO Mol Med 2013; 5: p.949-966.
45. Marie C, Pitton C, Fitting C, Cavaillon JM. *Regulation by anti-inflammatory cytokines(IL-4, IL-10, IL-13 and TGFb) of interleukin-8 production by LPS- and/or TNFa-activated human polymorphonuclear cell.* Med Inflamm 1996; 5: p334-340.

Chapter 4: Regulation of *CPB2* expression in THP-1 macrophages

4.1 Introduction

Normal haemostasis is characterized by the appropriate regulation of coagulation and fibrinolysis, preventing excessive blood loss following vascular injury while maintaining the fluidity of the blood at sites remote from the injury. The role of the coagulation cascade is to generate fibrin, the major structural component of the blood clot, which is converted from soluble fibrinogen to insoluble fibrin. The goal of the fibrinolysis cascade is to convert insoluble fibrin to soluble fibrin degradation products, leading to the dissolution of the clot. Thrombin-activatable fibrinolysis inhibitor (TAFI, Human Genome Organization gene name carboxypeptidase B2, symbol *CPB2*), is a plasma protein that has been demonstrated to play key roles in controlling this balance through attenuation of fibrinolysis [1]. TAFI is a plasma zymogen that upon activation by thrombin [2], the thrombin-thrombomodulin complex [3] or plasmin [4], becomes an enzyme TAFIa, with basic carboxypeptidase activity. TAFIa cleaves carboxy-terminal arginine and lysine residues from various protein and peptide substrates. The discovery of TAFI by several independent groups led to the variation in nomenclature, such that TAFI is also known as plasma procarboxypeptidase B [5], procarboxypeptidase R (for arginine) [6], or procarboxypeptidase U (for unstable) [7]. TAFIa attenuates fibrinolysis by removing carboxy-terminal lysine residues from partially degraded fibrin, leading to interruption of multiple positive feedback mechanisms in the fibrinolysis cascade [1, 8-10].

Plasma TAFI levels vary substantially in the human population, ranging from 100 to 300 nM [11]. The significance of this variability lies in the fact that the K_m for activation of TAFI by thrombin is 1μM [3], which is well above the physiological

concentrations of TAFI. An individual with higher TAFI zymogen levels would thus produce more TAFIa enzyme for any given thrombogenic stimulus. Accordingly, several clinical studies have established that elevated TAFI levels or markers of its activation have been associated with increased risk for the development of various thrombotic and atherothrombotic disorders, such as venous thrombosis [12, 13], recurrent venous thromboembolism [14], ischaemic stroke [15-17] and coronary heart disease [18], as well as the risk of cardiovascular death [19].

In addition to acting as a molecular link between coagulation and fibrinolysis, the TAFI pathway may also mediate molecular connections between coagulation and inflammation. Undoubtedly, coagulation and inflammation are intimately connected in the sense that vascular injury leads to the activation of both. TAFIa mediates the interplay between coagulation and inflammation by inactivating several pro-inflammatory peptides, such as the anaphylatoxins C3a and C5a [20], osteopontin [21] and bradykinin [22, 23], and by activating anti-inflammatory mediators, such as the adipokine chemerin [24]. Recent evidence suggests that TAFIa is capable of modulating the plasminogen-plasmin system by attenuating pericellular plasminogen activation [25, 26]. Activation of plasminogen to plasmin is achieved by plasminogen activators such as t-PA and u-PA, and the precise spatial and temporal control for the generation of plasmin is carefully orchestrated by the balance between plasminogen activation and plasmin inhibition. The latter can occur either directly, by α2-antiplasmin, or indirectly, by inactivation of plasminogen activators, t-PA and u-PA. Binding of plasminogen to cell surface receptors is an example of a mechanism for the spatial control of plasmin generation. Plasmin generated at the surface of cells activates matrix metalloproteinases (MMPs), hydrolyzing extracellular proteins (such as fibrin), proteins of the extracellular matrix (ECM), and

activating growth factors (such as the latent transforming growth factor β, TGF-β). TAFIa can cleave carboxy-terminal lysine residues from cell surface receptors, thus attenuating generation of plasmin at the cell surface. Attenuation of pericellular plasminogen activation by TAFIa is expected to have pro-thrombotic consequences in the context of the vascular wall, in keeping with the role of TAFIa as an anti-fibrinolytic agent. Additionally, this action of TAFIa would result in the reduction of activation of TGF-β, in the absence of which cytokines might induce smooth muscle cell proliferation and the transformation of these cells to a more atherogenic cellular phenotype [27, 28].

During atherogenesis, the plaque progression is accompanied by secretion of growth factors and cytokines by macrophages. Additionally, plaque-resident macrophages uptake the surrounding lipid molecules via scavenger receptors, leading to formation of foam cells, which in turn stimulate vascular smooth muscle cell growth and interstitial collagen synthesis [29]. Various atherogenic factors, such as Lp(a), induce the expression of adhesion molecules, ICAM-1 and Mac-1 integrin, contributing to endothelial dysfunction by promoting adhesion of inflammatory cells and differentiation of monocytes into macrophages, increasing the pool of foam cells. Endothelial dysfunction is also accompanied by reduced NO bioavailability and increased oxidant excess [30], which contribute not only to initiation but also to progression of atherosclerotic plaque formation and triggering of cardiovascular events. The plasminogen-plasmin system also becomes deranged, through stimulation of PAI-1 production by endothelial cells by Lp(a), thus decreasing plasmin formation, consequence of which may also be attributed by the action of TAFIa in this context. The events that occur in the vessel wall during atherogenesis also have a pro-inflammatory component, namely the production of cytokines by vascular cells, and through various autocrine and paracrine mechanisms,

expression of adhesion molecules by the endothelium and the accompanying monocyte chemotaxis, and the proliferation of smooth muscle cells.

That TAFIa can modulate pericellular plasminogen activation *in vivo* has been demonstrated in TAFI-deficient ($TAFI^{-/-}$) mice with hemizygous plasminogen background (thus possessing half the amount of plasminogen compared to their wild-type counterparts). Following peritoneal thioglycollate injection, TAFI-deficient mice demonstrated increased leukocyte migration to the peritoneum, in keeping with the role of TAFIa in attenuation of pericellular plasminogen activation [25].

Expression of *CPB2* in the liver is the major contributor to the plasma pool of TAFI. However, recently evidence suggests that *CPB2* is expressed at sites outside the liver. The initial report for this phenomenon described the existence of a platelet pool of TAFI that can be released upon platelet activation [31]. The data generated by our laboratory contributed significantly to this field of TAFI research. We identified expression of *CPB2* in vascular and inflammatory cell types, and found *CPB2* mRNA in the human megakaryoblastic cell lines MEG-01 and Dami, the human monocytoid cell line THP-1 as well as THP-1 cells differentiated into a macrophage-like phenotype, and in primary human umbilical vein and coronary artery endothelial cells [32]. *CPB2* mRNA abundance in MEG-01, Dami, and THP-1 cells was modulated by the state of differentiation of these cells. While *CPB2* mRNA abundance was higher in differentiated Dami and differentiated MEG-01 cells, in case of THP-1 cells differentiation was accompanied by a decrease in *CPB2* mRNA abundance. The significance for the modulation of mRNA abundance in these cell types is presently unknown, but nonetheless warrants further investigation. In fact, the regulatory mechanisms of *CPB2* expression that operate in extra-hepatic tissues still remain elusive. Since macrophages

infiltrate diseased vessel walls and are subject to stimulation by inflammatory mediators, it is expected that the regulation of *CPB2* expression could impact regulation of the plasminogen system locally, with all the attendant downstream effects. In the current investigation we set out to examine the regulatory mechanisms of *CPB2* expression in THP-1 macrophages, as they are a cell type that contributes importantly to the physiological and pathological processes of vascular biology, in order to gain further insights into the novel roles of the TAFI pathway beyond clot lysis.

4.2 Experimental procedures

4.2.1 Mammalian cell culture

Cells were maintained in a humidified incubator at 37°C under a 95% room air/5% CO2 atmosphere. THP-1 monocytes were cultured as previously described [32]. For TAFIa assay experiments, THP-1 monocytes were seeded in 60-mm culture dishes and differentiated into macrophages by incubation with PMA (1 nM final concentration) for 72 hours. PMA was removed and normal growth media was added lacking serum. Cells were incubated in serum-deficient media with pro-inflammatory cytokines TNFα (50 pg/ml), IL-8 (30ng/ml) , IL-6 (10ng/ml) in combination with IL-1β (1ng/ml), LPS (1μg/ml) and IL-10 (1ng/ml), for up to 48 hours. Conditioned media was collected as well as the total cellular lysates in lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA). TAFI was quantitatively activated with thrombin-thrombomodulin prior to a functional assay specific for TAFIa (described below).

4.2.2 RNA isolation and qRT-PCR

THP-1 monocytes were seeded in 12-well plates and differentiated by incubation with PMA (1 nM final concentration) for 72 hrs. PMA was removed and the cells were

maintained in normal growth media thereafter. Inflammatory mediators were added, where appropriate, at the following concentrations: TNFα (50 pg/mL), combination of IL-6 (10 ng/mL) and IL-1β (1 ng/mL), IL-8 (30 ng/mL), LPS (1 μg/mL), IL-10 (1 ng/mL) or IL-13 (10 ng/mL). For *CPB2* mRNA quantitation experiments, the cells were incubated with treatments for 24 hours. For mRNA stability experiments, Actinomycin D was added to arrest transcription, to a final concentration of 1μM, and RNA was harvested at various time points thereafter, up to 6 hours. RNA was extracted with TriZol reagent (Invitrogen) and genomic DNA was removed by incubation with DNaseI (Promega) for 2 hours at 37^0C. Endogenous *CPB2* mRNA was quantitated using absolute qRT-PCR with appropriate primer and probe sets, using Brilliant III qRT-PCR kit (Agilent Technologies), with 2 μg of total RNA per each qRT-PCR reaction. For endogenous *CPB2* mRNA quantitation, primer and probe set that recognizes the region common to all three polyadenylated forms (named 1660) was used, forward primer: 5'-GAC CAC CCT TCC TTT TGT TGA GT-3'; reverse primer : 5'-GGGTGGTCA GAA GTA CAT TAA AGATTT-3'; probe sequence: 5'-/56-FAM/TGT GCCTTT /ZEN/AGA AAT ACA ACC ATG CAT TCC G/3IABkFQ/-3' (where 6-FAM is the fluorescent dye and ZEN and Iowa Black FQ [3IaBkFQ] are quenchers). This primer and probe set was used to quantitate *CPB2* transcript for mRNA stability experiments as well. A set of appropriate standards were included with each qRT-PCR run, and the amount of transcript was determined using the equation of the standard curve. To determine the amounts of each of the three polyadenylated forms of *CPB2* mRNA, two more primer-probe sets were designed. The 1693 primer-probe set recognizes the region common to the intermediate and the longest polyA species, forward primer: 5'-CTG GGC CAT CGC CTA ATT AC-3'; reverse primer: 5'-GGGTGGTCA GAA GTA CAT TAA AGATTT-3'; probe sequence: 5'-/56-

FAM/TGT GCCTTT /ZEN/AGA AAT ACA ACC ATG CAT TCC G/3IABkFQ/-3'. The last prime- probe set, 1819 was designed to only recognize the longest polyA species, forward primer: 5'-TAC AAC CAT GCATTC CGT TTG-3'; reverse primer: 5'-TTG ACA CCC CTC CCCTTT C-3'; probe sequence: 5'-/56-FAM/TCC ACG GTA /ZEN/ATT AGG CGATGG CCC /3IABkFQ/-3'. The amount of each polyA form was therefore determined by subtracting the 1693 and 1819 signals from the 1660 signal (for the amount of the shortest form), by subtracting the 1819 signal from the 1693 signal (for the amount of the intermediate form) and the amount of the longest polyA form was obtained directly from the 1819 signal. The number of transcripts were then expressed as percentage of total.

4.2.3 TAFI activation and TAFIa assay

A highly sensitive functional assay for TAFIa was implemented as previously described [33]. The first step is quantitative activation of all TAFI present in the sample, which was carried out as described previously [34]. TAFIa concentrations measured by the TAFIa assay in each sample were divided by the respective total cellular protein values obtained using the BCA assay (Pierce Chemical) to account for the differences in cell numbers. Results were then normalized to the untreated medium sample for each time point, expressed as fraction per mg of total cellular protein.

4.3 Results

4.3.1 Pro-inflammatory mediators increase TAFI protein levels

The effects of inflammatory mediators on the expression of TAFI in human macrophages were examined using differentiated THP-1 cells as a model system, with a recently developed assay specific for TAFIa [33] . The cells were treated with pro-inflammatory cytokines and mediators: TNFα, IL-6 in combination with IL-1β, and IL-8,

LPS, as well as the anti-inflammatory cytokine, IL-10. THP-1 monocytes were differentiated using phorbol-12-myristate-13-acetate (PMA) for 72 hours, the inflammatory mediators were added to the culture medium, and the conditioned medium was collected thereafter at various time points for quantification of TAFI protein using the TAFIa assay. The increase in TAFI protein levels was observable as early as 12 hours post-treatment, albeit to an extent that was not statistically significant. Statistically significant elevation of TAFI levels occurred at later time points. After 24 hours, the increase ranged from less than 10% in case of TNFα and IL-10, 14% for IL-6 and IL-1β combination and for IL-8, and up to 64% for LPS-treated cells (Figure 4.1). The capacity of these mediators to modulate TAFI protein continued even after 24 hours of treatment, and the increase in TAFI levels culminated at 44% for TNFα, 63% for IL-8 and 69% IL-6 and IL-1β -treated cells. The maximal effect was observed with LPS, with an increase of 109% and IL-10 treatment resulted in 90% increase in TAFI protein.

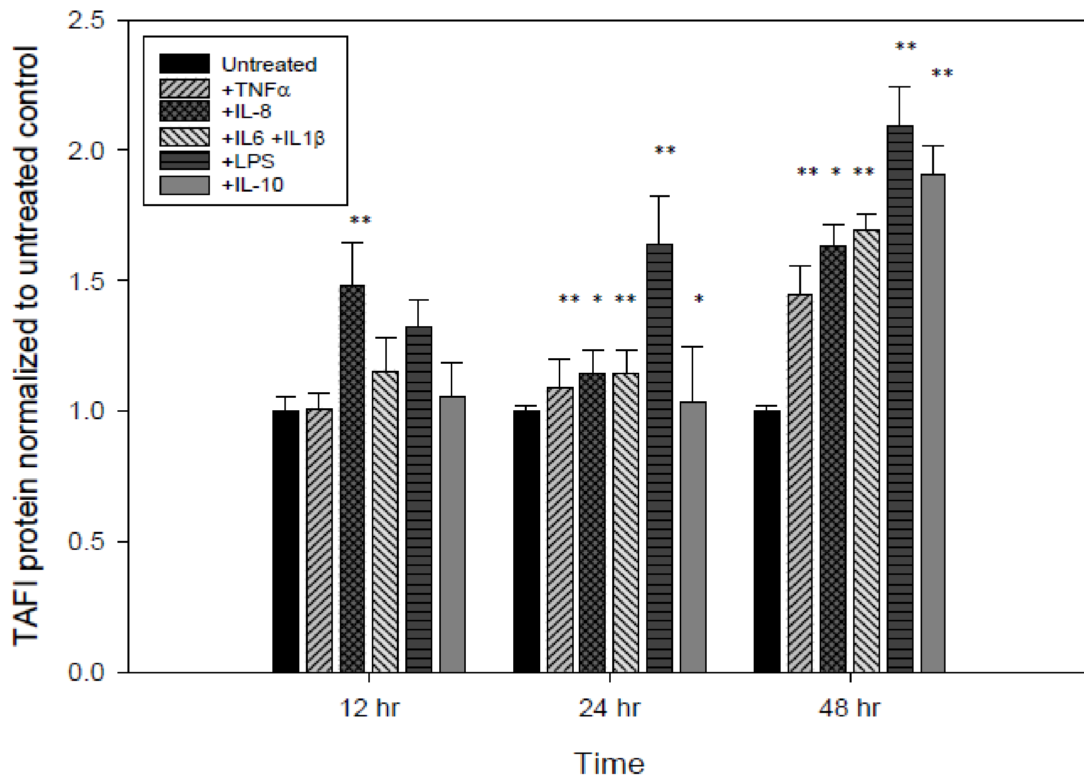

Figure 4.1 Inflammatory mediators modulate TAFI protein levels in THP-1 macrophages. THP-1 monocytes were seeded in 60-mm culture dishes and differentiated into macrophages by incubation with PMA (1 nM final concentration) for 72 hours. PMA was removed and normal growth media was added lacking serum. Cells were incubated in serum-deficient media with pro-inflammatory cytokines TNFα (50 pg/ml), IL-8 (30ng/ml) , IL-6 (10ng/ml) in combination with IL-1β (1ng/ml), LPS (1μg/ml) and IL-10 (1ng/ml). Conditioned media was collected at various time points and TAFI was quantitatively activated with thrombin-thrombomodulin prior to a functional assay specific for TAFIa. TAFIa amounts in each sample are corrected to their corresponding total cellular protein content and expressed relative to the TAFIa amount present in the untreated samples at the respective time points. The data shown are the mean ± s.e.m. of three independent experiments. *: $p < 0.05$; ** $p < 0.01$ versus untreated control by Student's t-test.

4.3.2 Effects of pro- and anti-inflammatory cytokines and mediators on *CPB2* mRNA abundance

We next set out to determine whether the changes observed at the protein levels are reflected on *CPB2* mRNA levels upon treatment of THP-1 macrophages with the inflammatory mediators. We measured endogenous *CPB2* mRNA levels with real-time qRT-PCR following 24-hour incubation with the inflammatory mediators. All inflammatory cytokines under study modulated *CPB2* mRNA levels in a way that is reflective of modulation for TAFI protein levels. *CPB2* mRNA abundance was increased in cells treated with TNFα and LPS by 13%, IL-6/IL-1β 33%, IL-8 by 37%, and IL-10 by 21% (Figure 4.2). However, the results did not reach statistical significance for TNFα and IL-6/IL-1β treatments.

4.3.3 Effects of pro- and anti-inflammatory cytokines and mediators on *CPB2* mRNA stability

In a recently published report by our laboratory we described the expression of *CPB2* in vascular and inflammatory cell types, including THP-1 macrophages [32]. We observed that *CPB2* mRNA abundance is 5000-fold lower than that in HepG2 cells. Moreover, we also quantitated the amount of TAFI protein and found that THP-1 macrophages produce seven times less TAFI protein per million cells compared to HepG2 cells. In the present study, we measured endogenous *CPB2* mRNA half-life in THP-1 macrophages, and found it to be significantly lower than that in HepG2 cells. Endogenous *CPB2* mRNA half-life in HepG2 cells is 3.1 hours [35], and it is 33% lower in THP-1 macrophages (2.1 hours). This is to our knowledge the first report measuring half-life of endogenous *CPB2* mRNA in THP-1 macrophages.

The modulation of *CPB2* mRNA abundance upon treatment of THP-1 macrophages with the inflammatory mediators prompted us to examine whether concomitant changes also occur in mRNA stability. THP-1 monocytes were differentiated to macrophages with a 72-hour-long PMA treatment, and the cells were then incubated with the inflammatory mediators for 24 hours. Actinomycin D was then added to the culture medium in the absence of the inflammatory mediators to arrest transcription and cell lysates were harvested at various time points thereafter, up to 6 hours. RNA was extracted and subjected to real-time qRT-PCR analysis, using primers and probes specific for human TAFI. Linear regression analysis was performed and mRNA half-lives were determined (Figure 4.3). Treatments with IL-8, LPS and IL-6 together with IL-1β resulted in 30% stabilization of *CPB2* mRNA, while TNFα treatment caused only a mild increase (17%) in mRNA half-life (Figure 3, Table 1). IL-10 treatment resulted in stabilization of *CPB2* mRNA by 26% (Figure 4.3, Table 4.1). These changes in mRNA stability are in line with the observations of TAFI protein secretion and *CPB2* mRNA abundance in THP-1 macrophages. However, the results did not reach statistical significance for TNFα and IL-6/IL-1β treatments. Therefore, the observed effects of TNFα, IL-8, IL6 in combination with IL1β, LPS and IL-10 on TAFI protein levels and *CPB2* mRNA abundance in THP-1 macrophages appear to be, at least in part, attributable to concomitant changes in mRNA stability.

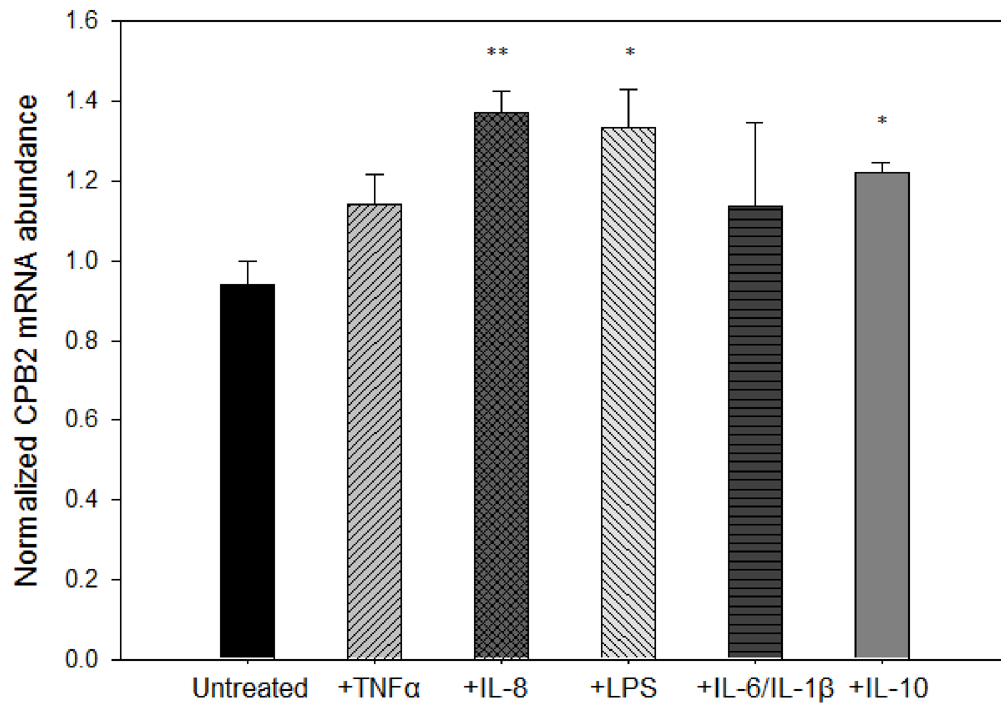

Figure 4.2 Inflammatory mediators modulate abundance of endogenous *CPB2* mRNA in THP-1 macrophages. THP-1 monocytes were seeded in 12-well plates and differentiated into macrophages by incubation with PMA (1 nM final concentration) for 72 hours. PMA was removed and normal growth media was added. THP-1 macrophages were treated with indicated inflammatory mediators for 24 hours, followed by isolation of total RNA and DNase I digestion. Endogenous *CPB2* mRNA was quantitated with real-time qRT-PCR using Taq-based chemistry in a multiplex reaction containing primer and probe sets specific for human TAFI and GAPDH. The relative abundance of TAFI mRNA for each sample was then normalized to untreated control. The data represents the average of two independent experiments. *: $p < 0.5$; ** $p < 0.02$ versus untreated control by Student's t-test.

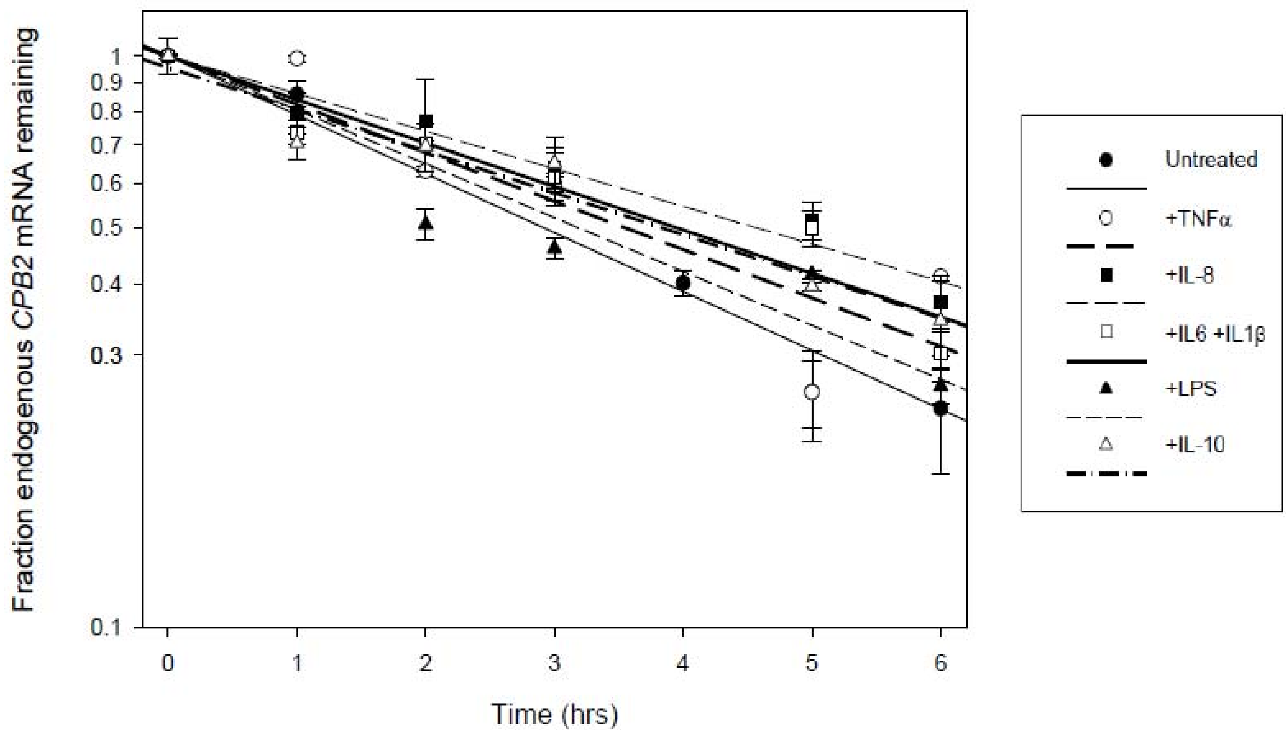

Figure 4.3 Stability of endogenous *CPB2* mRNA is modulated by inflammatory mediators in THP-1 macrophages. THP-1 monocytes were seeded in 12-well plates and differentiated into macrophages by incubation with PMA (1 nM final concentration) for 72 hours. PMA was removed and normal growth media was added. Cells were then treated with inflammatory mediators for 24 hours. Actinomycin D was added to the final concentration of 5μg/ml in the absence of the treatments. The cell lysates were collected at various time points thereafter, up to 6 hours. RNA was extracted and subjected to absolute real-time qRT- PCR analysis, using primer and probe sets specific for human TAFI. Linear regression analysis was performed and *CPB2* transcript half-lives were determined (Table 3.1). The data represents the average of two independent experiments.

Table 4.1 Stability of *CPB2* mRNA in THP-1 macrophages after cytokine treatment

Treatment	$t_{(1/2)}$ (hrs)
Untreated	2.1±0.2
+TNFα	3.7 ±1.2
+IL-8	2.8±0.2*
+IL-6/IL-1β	2.8±0.7
+LPS	2.7±0.1*
+IL-10	2.6±0.2*

*: $p < 0.05$ versus untreated control by Student's t-test.

4.3.4 Effects of inflammatory mediators on polyadenylation site selection in THP- 1 macrophages

CPB2 3'-flanking region contains 3 possible polyadenylation (polyA) sites at positions +1660, +1693 and +1819 (from the stop codon), resulting in 3'-UTRs of 3 different lengths. We have previously determined that polyadenylation site selection appears to be a mechanism that is involved in regulated changes in *CPB2* mRNA abundance through modulation of *CPB2* mRNA stability in HepG2 cells [36]. Treatment of HepG2 cells with a combination of IL-6 and IL-1β resulted in 60% decrease in *CPB2* mRNA abundance owing the preferential formation of the longest transcript that is further destabilized by a factor of two; stabilities of the intermediate and the shortest transcripts remained unchanged. Moreover, in the absence of inflammatory mediators the longest transcript only accounts for about 1% of the total pool of *CPB2* transcripts, whereas the shortest transcript is the most abundant, followed by the intermediate-length transcript. Therefore, polyA site selection appears to be modulated in the presence of inflammatory mediators in HepG2 cells. Here we investigated whether the same paradigm extends to THP-1 macrophages.

First we examined the distribution of the three differentially polyadenylated *CPB2* forms under normal conditions. Unlike HepG2 cells, in which the shortest form is the most abundant, the majority of the total pool of *CPB2* transcripts is comprised of the longest polyA form in THP-1 macrophages (47%), followed by the shortest form (41%) and then the intermediate form (12%) (Figure 4.4). Upon treatment of THP-1 macrophages with the combination of cytokines IL-6 and IL-1β, we observed a shift in the polyadenylation site selection, albeit to a much less dramatic extent. In HepG2 cells, this treatment resulted in almost exclusive production of the longest polyadenylation form

(98%) [36], while in THP-1 macrophages, the longest transcript now comprises 40% of the total pool of *CPB2* transcripts (Figure 4.4). In addition, the shortest transcript was more favourably produced and comprised 60% of the total pool, while the intermediate form was virtually absent. Therefore, polyA site selection may also be modulated in THP-1 macrophages, albeit to a lower extent than what we observed in HepG2 cells upon treatment of cells with IL-6 and IL-1β. We then examined the effect of other inflammatory mediators on polyadenylation site selection and observed a similar trend to that produced by IL6/IL1β. Generally, inflammatory mediators caused a mild shift towards the production of the shortest polyadenylation form, and the longest polyA form seems to be less favourable produced in the presence of these treatments (Figure 4.5).

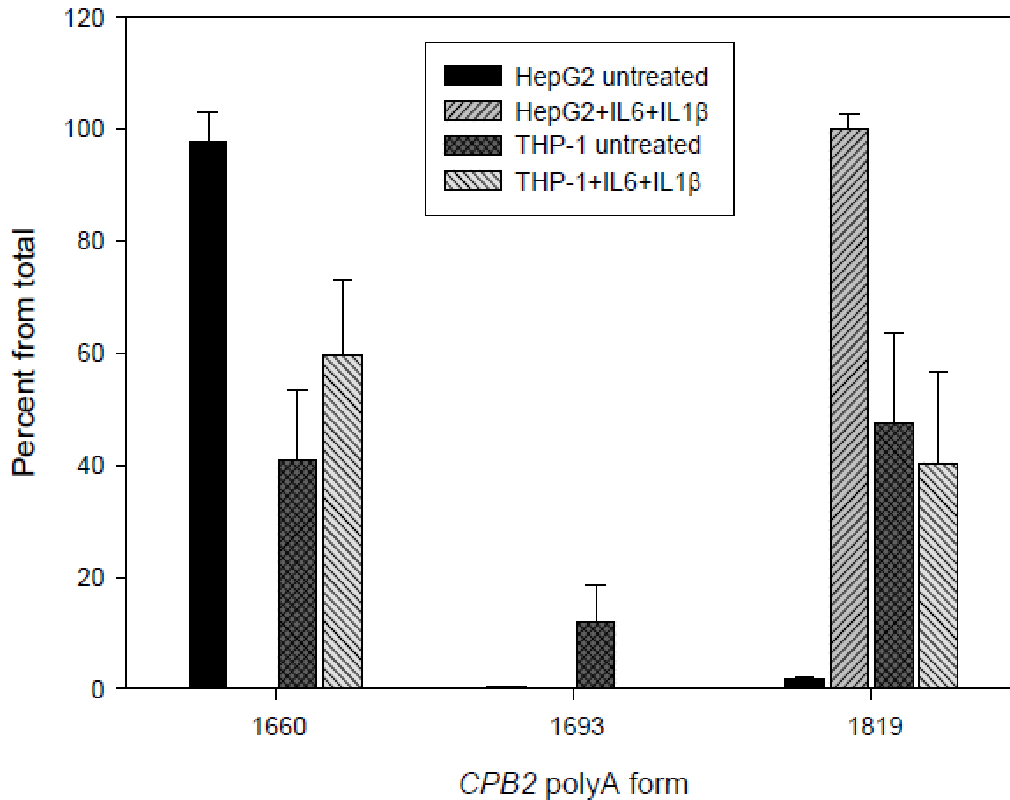

Figure 4.4 Distribution of three differentially polyadenylated forms is different in HepG2 cells and THP- macrophages. THP-1 monocytes were differentiated in 6-well plates with PMA for 72 hours, followed by addition of IL-6 (10 ng/ml) and IL-1β (1 ng/ml) (where appropriate) for 24 hours. RNA was extracted and subjected to absolute real-time qRT-PCR using primer and probe sets specifically designed to detect the three differentially polyadenylated species of *CPB2.* Each polyadenylated form is expressed as percentage from total. The data represents the average of two independent experiments. The data for HepG2 cells are from ref. 36.

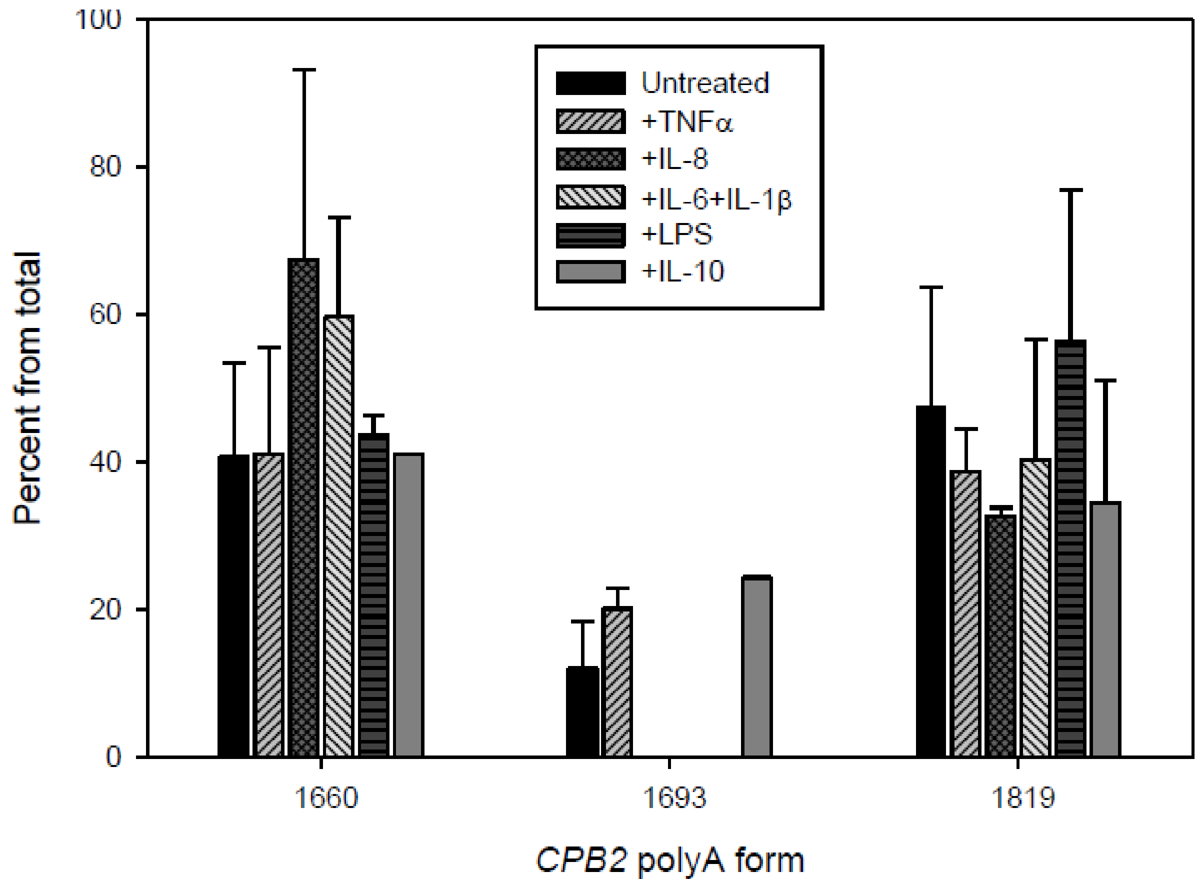

Figure 4.5 Polyadenylation pattern is modulated in THP-1 macrophages by inflammatory mediators. THP-1 monocytes were differentiated in 6-well plates with PMA for 72 hours, followed by addition of inflammatory mediators: TNFα (50 pg/ml), IL-8 (30ng/ml) , IL-6 (10ng/ml) in combination with IL-1β (1ng/ml), LPS (1μg/ml) and IL-10 (1ng/ml) (where appropriate) for 24 hours. RNA was extracted and subjected to absolute real-time qRT-PCR using primer and probe sets specifically designed to detect the three differentially polyadenylated species of *CPB2*. Each polyadenylated form is expressed as percentage from total. The data represents the average of two independent experiments.

4.4 Discussion

Human THP-1 cell line was established over 30 years ago [37]. These cells resemble primary monocytes in morphology and differentiation properties. After exposure to PMA, almost all THP-1 cells start to adhere to culture plates, accompanied by expression of macrophage-specific markers and the development of macrophage-like phenotype. Although the use of 25-dihydroxyvitamin D3 ($1\alpha,25(OH)_2D3$) has also been reported in the literature as an agent used to differentiate THP-1 cells into macrophages, PMA and 25-dihydroxyvitamin D3 regulate different signaling pathways [38]. PMA treatment results in a more mature phenotype with higher levels of adherence, accompanied by lower rate of proliferation and a higher rate of phagocytosis. THP-1 cells have been shown to resemble the primary monocytes isolated from healthy subjects and subjects with disease (such as diabetics [39] and chronic renal failure [40]), and these cells also mirror the *in situ* alteration of macrophages in adipose tissue from obese subjects [41] and in atherosclerotic lesions [42]. Therefore, PMA-differentiated THP-1 monocytes are generally used as a model system to study macrophage function. Key regulatory mechanisms that operate in monocytes and macrophages include regulation of mRNA stability, transcriptional control, post-translational modifications and protein degradation. That the inflammatory mediators, such as bacterial LPS, modulate transcriptional and post-transcriptional profiles in macrophages has been described previously in the literature [43-45]. Additionally, the involvement of microRNA (miR) pathway in fine-tuning the immune response in THP-1 macrophages has also been described. Upon LPS challenge, a marked upregulation of several miRs has been reported, namely miR-146a/b, miR-155, and miR-132, miR-214, miR-195a and miR-16 [40, 46-50]. We have recently discovered the expression of TAFI mRNA and secretion of

TAFI protein by THP-1 cells and the aim of the present investigation was to elucidate regulatory mechanisms that govern *CPB2* expression both under steady state conditions, and in the presence of inflammatory mediators.

Until the discovery of extra-hepatic TAFI expression by our group and others [31, 32, 51], the liver was thought to be the primary contributor to the plasma pool of TAFI. Indeed, expression of *CPB2* by the liver likely accounts for the primary source of plasma TAFI, as the levels of *CPB2* mRNA and TAFI protein are the highest in hepatocytes, compared to other cell types. However, in the context of a thrombus microenvironment, the contribution of TAFI protein by platelets and macrophages may reach physiologically significance, given the cell density at these sites. Therefore, even the subtle changes in TAFI protein production by these cells within the thrombus or in the vessel wall might appreciably affect the fibrinolytic capacity.

We observed that the inflammatory mediators TNFα, IL-8, IL-6 in combination with IL-1β, LPS and IL-10 increased TAFI protein secretion by THP-1 macrophages, and this effect was also reflected in *CPB2* mRNA abundance. Since the expression of *CPB2* mRNA is 5000-fold lower in THP-1 macrophages compared to HepG2 cells [32], we were unable to study the effects of these mediators on *CPB2* promoter activity, as the basal activity is below the detection limit of commonly used assays (i.e. luciferase reporter assay). However, that the inflammatory mediators act through transcriptional mechanism is unlikely, since they classically employ the activation of NFκB pathway, followed by its binding to the NFκB binding site within the responsive promoters. Previous study within our laboratory was conducted to characterize the human *CPB2* promoter and to identify transcription factors that regulate promoter activity [52]. This study revealed that the consensus NFκB binding site is absent from the human *CPB2*

promoter. In fact, we identified the presence of this site in the *CPB2* promoter encoding murine TAFI, whereas the human counterpart was found to contain substitutions of key nucleotides, thus altering the core consensus from GGGAC to GGGTT. Therefore, these substitutions likely render this site non-functional within the human promoter. The changes in *CPB2* mRNA abundance therefore are likely consequence of changes in *CPB2* mRNA stability, as we have previously identified the key role of *CPB2* 3'-UTR in mediating mRNA stability under steady state conditions and in the presence of inflammatory mediators in hepatocytes. Therefore, this control of mRNA stability might extend to THP-1 macrophages as well.

While inflammatory mediators were found to decrease *CPB2* mRNA abundance and stability in HepG2 cells (unpublished data), they exert the opposite effect on mRNA stability in THP-1 macrophages, resulting in stabilization and an increase in total abundance of *CPB2* mRNA upon 24 hour incubation with THP-1 macrophages. This stabilization ranged from a mild 17% in case of TNFα, to 26% in case of IL-10, and up to 30% in the presence of IL-8, LPS and IL-6/IL-1β combination treatment. Therefore, *CPB*2 3'-UTR appears to mediate regulated changes in mRNA stability differently in hepatocytes and THP-macrophages. This difference likely arises from the difference in profiles of post-transcriptional proteome and microRNA-ome in these 2 cell types. RNA-binding protein factors, such as tristetraprolin (TTP), AUF1, BRF1, and HuR may be subjected to different control mechanisms in different cell types [53]. Since macrophages are a cell type directly involved in inflammation, this specialized function is likely accompanied by a specific set of regulatory mechanisms that is different than that in hepatocytes. Liver certainly plays a role in inflammation, but is often the site of inflammation rather than the initiator of the inflammatory process. Macrophages are

known to infiltrate the sites of inflammation, and this results in a switch from an anti-inflammatory to a pro-inflammatory function, followed by pro-inflammatory cytokine production by these cells.

The gene encoding ATP binding cassette protein A1 (ABCA1) is subjected to differential regulation in expression in hepatocytes and macrophages [54]. ABCA1 gene was found to be regulated differently in these two cell types, and the authors concluded that this difference likely occurs at the post-transcriptional level. Niemann-Pick type C1 (Npc1) protein inactivation results in lipid accumulation in late endosomes and lysosomes, leading to a defect of Abca1-mediated lipid efflux to apolipoprotein A-I (apoA-I) in macrophages and fibroblasts. Here the authors investigated the role of Npc1 in ABCA1-mediated lipid efflux to apoA-I in hepatocytes, the major cell type contributing to HDL formation. They concluded that the increased ABCA1 levels are largely due to changes in post-transcriptional control in hepatocytes, mainly through increased translation rate, an event that was not observed in macrophages. This increase in translation rate was mediated by Cathepsin D, which was identified as a positive modulator of ABCA1. It was markedly increased at both mRNA and protein levels by Npc1 inactivation in hepatocytes but not in macrophages. Therefore, *CPB2* gene expression regulation may also be subjected to tissue-specific regulatory mechanisms in hepatocytes and macrophages in the presence of a common stimulus.

Unlike HepG2 cells in which the shortest form is the most abundant, the majority of the total pool of *CPB2* transcripts is comprised of the longest polyadenylated form in THP-1 macrophages (47%), followed by the shortest form (41%) and then the intermediate form (12%). Upon treatment of THP-1 macrophages with the combination of cytokines IL-6 and IL-1β, we observed a mild shift in polyadenylation site selection, to a

much less dramatic extent compared to HepG2 cells. In HepG2 cells, this treatment resulted in almost exclusive production of the longest polyadenylation form (98%), while in THP-1 macrophages, the longest transcript comprises 40% of the total pool of *CPB2* transcripts. In addition, the shortest transcript was more favourably produced and now comprised 60% of the total pool, while the intermediate form was virtually absent. Therefore, polyA site selection also appears to be modulated in THP-1 macrophages, the extent of which is much milder than what we observed in HepG2 cells upon treatment of cells with IL-6 and IL-1β. We then examined the effect of other inflammatory mediators on polyA site selection and observed a similar trend to that produced by IL6/IL1β. Generally, inflammatory mediators cause a mild shift towards the production of the shortest polyA form, and the longest polyA form seems to be less favourably produced in the presence of these inflammatory mediators.

The mechanism of 3'end processing of nascent transcripts in eukaryotic cells involves endonucleolytic cleavage and synthesis of a polyA tail, which are dictated by specific signals embedded in the transcript. (reviewed in [55]). Mutations affecting polyA site usage have been implicated in several human diseases (reviewed in [56]), such as thrombophilia and some thalassemias, underlining the importance of proper 3′-end processing in gene expression regulation and its relevance to human health. Numerous genomic studies have uncovered widespread occurrences of alternative polyadenylation in metazoan protein-coding mRNAs: 70–79% of mammalian genes [57, 58] and about half of the genes in flies [59], worm [60], and zebrafish [61] have been reported to be regulated by alternative polyadenylation. Due to differences in experimental conditions and bioinformatic methods, a consensus has not been reached as to the exact statistic of the extent of alternative polyadenylation. Nonetheless, it has become clear that a large

number of eukaryotic genes express isoforms that display significant differences in the 3’-portion of the transcript. The functional significance of alternative polyadenylation can be appreciated from the fact that the 3′-UTR plays a key role in regulating mRNA metabolism, mainly through the existence of the embedded *cis* elements, including its localization, stability, and translation. Therefore, the transcript isoforms arising due to alternative polyadenylation have different 3′-UTR lengths and hence distinct properties.

We have identified a role of alternative polyadenylation in mediating regulated changes in *CPB2* mRNA abundance in HepG2 cells previously [36]. In the present study, we investigated whether the same regulatory paradigm extends to THP-1 macrophages. While the first polyA signal was found to be used most frequently used in HepG2 cells under normal conditions, such is not the case for THP-1 macrophages; in these cells, the most distal polyA site was found to be used most favourably, followed by the first and the second polyA sites. Upon treatment of cells with the combination of inflammatory cytokines IL-6 and IL-1β, in HepG2 cells a dramatic shift towards the production of the longest transcript was observed, so as to almost exclusively result in the production of this polyadenylated form. However, a shift towards the production of the shortest polyA form was observed upon treatment of THP-1 macrophages with the same cytokine combination. Since the region between the second and the third polyA sites contains an instability element, it is tempting to speculate that the inclusion of this region (such as occurs in HepG2 cells) is the main driving mechanism behind destabilization of *CBP2* transcripts, resulting in reduction of total abundance of *CPB2* mRNA under these inflammatory conditions. Conversely, more favourable production of the shortest transcript in THP-1 macrophages is accompanied by exclusion of the last instability element, resulting in stabilization of the *CPB2* mRNA and an increase in overall

abundance in the presence of IL-6 and IL-1β. Therefore, the same stimulus appears to produce different polyadenylation patterns in HepG2 cells and THP-1 macrophages, resulting in concomitant decrease or increase in TAFI protein produced, respectively.

In summary, this investigation uncovered novel mechanisms of *CPB2* gene expression regulation in THP-1 macrophages. We observed that the inflammatory mediators TNFα, IL-8, IL-6 in combination with IL-1β, LPS and IL-10 increased TAFI protein production by THP-1 macrophages, and this effect was also reflected in *CPB2* mRNA abundance, owing to stabilization of the transcript under these conditions. Additionally, we investigated the distribution of the three differentially polyadenylated species in THP1 macrophages and found that the longest form is most favourably produced, followed by the shortest and the intermediate forms. Moreover, the pattern of alternative polyadenylation was modified in the presence of inflammatory mediators, resulting in a preferential formation of the shortest transcript. This may account for the observed stabilization of *CPB2* mRNA, and an increase in overall abundance in the presence of these inflammatory mediators. Taken together, these data suggest that *CPB2* gene expression regulation may be a subject to tissue specific control. Indeed, a growing number of RNA-binding proteins (RBPs) have been found to regulate cleavage and polyadenylation (reviewed in [62]). Given the tissue-specific expression of some RBPs, such proteins may play roles in defining tissue-specific alternative polyadenylation [53]. Therefore, it is of utmost importance to pinpoint the exact regulatory mechanisms that are subjected to different control in HepG2 cells and THP-1 macrophages to gain further insights into both gene expression regulation of *CPB2* as well as the more fundamental mechanisms of mRNA metabolism that operate in mammalian cells.

4.5 References

1. Nesheim, M., *Thrombin and fibrinolysis.* Chest, 2003. **124**(3 Suppl): p. 33S-9S.
2. Bajzar, L., R. Manuel, and M.E. Nesheim, *Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor.* J Biol Chem, 1995. **270**(24): p. 14477-84.
3. Bajzar, L., J. Morser, and M. Nesheim, *TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex.* J Biol Chem, 1996. **271**(28): p. 16603-8.
4. Mao, S.S., et al., *Characterization of plasmin-mediated activation of plasma procarboxypeptidase B. Modulation by glycosaminoglycans.* J Biol Chem, 1999. **274**(49): p. 35046-52.
5. Eaton, D.L., et al., *Isolation, molecular cloning, and partial characterization of a novel carboxypeptidase B from human plasma.* J Biol Chem, 1991. **266**(32): p. 21833-8.
6. Campbell, W. and H. Okada, *An arginine specific carboxypeptidase generated in blood during coagulation or inflammation which is unrelated to carboxypeptidase N or its subunits.* Biochem Biophys Res Commun, 1989. **162**(3): p. 933-9.
7. Hendriks, D., et al., *Characterisation of a carboxypeptidase in human serum distinct from carboxypeptidase N.* J Clin Chem Clin Biochem, 1989. **27**(5): p. 277-85.
8. Wang, W., et al., *A study of the mechanism of inhibition of fibrinolysis by activated thrombin-activable fibrinolysis inhibitor.* J Biol Chem, 1998. **273**(42): p. 27176-81.
9. Schneider, M. and M. Nesheim, *A study of the protection of plasmin from antiplasmin inhibition within an intact fibrin clot during the course of clot lysis.* J Biol Chem, 2004. **279**(14): p. 13333-9.
10. Schneider, M., et al., *Activated thrombin-activatable fibrinolysis inhibitor reduces the ability of high molecular weight fibrin degradation products to protect plasmin from antiplasmin.* J Biol Chem, 2004. **279**(14): p. 13340-5.
11. Frere, C., et al., *Quantification of thrombin activatable fibrinolysis inhibitor (TAFI) gene polymorphism effects on plasma levels of TAFI measured with assays insensitive to isoform-dependent artefact.* Thromb Haemost, 2005. **94**(2): p. 373-9.
12. van Tilburg, N.H., F.R. Rosendaal, and R.M. Bertina, *Thrombin activatable fibrinolysis inhibitor and the risk for deep vein thrombosis.* Blood, 2000. **95**(9): p. 2855-9.
13. Meltzer, M.E., et al., *Venous thrombosis risk associated with plasma hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1.* Blood, 2010. **116**(1): p. 113-21.
14. Eichinger, S., et al., *Thrombin-activatable fibrinolysis inhibitor and the risk for recurrent venous thromboembolism.* Blood, 2004. **103**(10): p. 3773-6.
15. Leebeek, F.W., et al., *High functional levels of thrombin-activatable fibrinolysis inhibitor are associated with an increased risk of first ischemic stroke.* J Thromb Haemost, 2005. **3**(10): p. 2211-8.

16. Rooth, E., et al., *Thrombin activatable fibrinolysis inhibitor and its relationship to fibrinolysis and inflammation during the acute and convalescent phase of ischemic stroke.* Blood Coagul Fibrinolysis, 2007. **18**(4): p. 365-70.
17. Ladenvall, C., et al., *Thrombin activatable fibrinolysis inhibitor activation peptide shows association with all major subtypes of ischemic stroke and with TAFI gene variation.* Arterioscler Thromb Vasc Biol, 2007. **27**(4): p. 955-62.
18. de Bruijne, E.L., et al., *The role of thrombin activatable fibrinolysis inhibitor in arterial thrombosis at a young age: the ATTAC study.* J Thromb Haemost, 2009. **7**(6): p. 919-27.
19. Tregouet, D.A., et al., *Activated thrombin activatable fibrinolysis inhibitor levels are associated with the risk of cardiovascular death in patients with coronary artery disease: the AtheroGene study.* J Thromb Haemost, 2009. **7**(1): p. 49-57.
20. Campbell, W.D., et al., *Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N.* Microbiol Immunol, 2002. **46**(2): p. 131-4.
21. Leung, L.L., T. Nishimura, and T. Myles, *Regulation of tissue inflammation by thrombin-activatable carboxypeptidase B (or TAFI).* Adv Exp Med Biol, 2008. **632**: p. 61-9.
22. Hendriks, D., et al., *Human serum carboxypeptidase U: a new kininase?* Agents Actions Suppl, 1992. **38 (Pt 1)**: p. 407-13.
23. Myles, T., et al., *Thrombin activatable fibrinolysis inhibitor, a potential regulator of vascular inflammation.* J Biol Chem, 2003. **278**(51): p. 51059-67.
24. Du, X.Y., et al., *Regulation of chemerin bioactivity by plasma carboxypeptidase N, carboxypeptidase B (activated thrombin-activable fibrinolysis inhibitor), and platelets.* J Biol Chem, 2009. **284**(2): p. 751-8.
25. Swaisgood, C.M., et al., *In vivo regulation of plasminogen function by plasma carboxypeptidase B.* J Clin Invest, 2002. **110**(9): p. 1275-82.
26. Morser, J., et al., *What has been learnt from the thrombin-activatable fibrinolysis inhibitor-deficient mouse?* J Thromb Haemost, 2010. **8**(5): p. 868-76.
27. Smith, E.B. and S. Cochran, *Factors influencing the accumulation in fibrous plaques of lipid derived from low density lipoprotein. II. Preferential immobilization of lipoprotein (a) (Lp(a)).* Atherosclerosis, 1990. **84**(2-3): p. 173-81.
28. Beisiegel, U., et al., *Lipoprotein(a) in the arterial wall.* Eur Heart J, 1990. **11 Suppl E**: p. 174-83.
29. Libby, P., *Inflammation in atherosclerosis.* Nature, 2002. **420**(6917): p. 868-74.
30. Aznar, J., et al., *Euglobulin clot lysis induced by tissue type plasminogen activator in subjects with increased levels and different isoforms of lipoprotein (a).* Thromb Res, 1993. **72**(5): p. 459-65.
31. Mosnier, L.O., et al., *Identification of thrombin activatable fibrinolysis inhibitor (TAFI) in human platelets.* Blood, 2003. **101**(12): p. 4844-6.
32. Lin, J.H., et al., *Identification of human thrombin-activatable fibrinolysis inhibitor in vascular and inflammatory cells.* Thromb Haemost, 2011. **105**(6): p. 999-1009.
33. Kim, P.Y., et al., *An assay for measuring functional activated thrombin-activatable fibrinolysis inhibitor in plasma.* Anal Biochem, 2008. **372**(1): p. 32-40.

34. Lin, J.H., et al., *The mRNA encoding TAFI is alternatively spliced in different cell types and produces intracellular forms of the protein lacking TAFIa activity.* Thromb Haemost, 2013. **109**(6): p. 1033-44.
35. Maret, D., et al., *Role of mRNA transcript stability in modulation of expression of the gene encoding thrombin activable fibrinolysis inhibitor.* J Thromb Haemost, 2004. **2**(11): p. 1969-79.
36. Boffa, M.B., et al., *Acute phase mediators modulate thrombin-activable fibrinolysis inhibitor (TAFI) gene expression in HepG2 cells.* J Biol Chem, 2003. **278**(11): p. 9250-7.
37. Tsuchiya, S., et al., *Establishment and characterization of a human acute monocytic leukemia cell line (THP-1).* Int J Cancer, 1980. **26**(2): p. 171-6.
38. Schwende, H., et al., *Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3.* J Leukoc Biol, 1996. **59**(4): p. 555-61.
39. Miao, F., et al., *Genome-wide analysis of histone lysine methylation variations caused by diabetic conditions in human monocytes.* J Biol Chem, 2007. **282**(18): p. 13854-63.
40. Li, L.M., et al., *Role of microRNA-214-targeting phosphatase and tensin homolog in advanced glycation end product-induced apoptosis delay in monocytes.* J Immunol, 2011. **186**(4): p. 2552-60.
41. Spencer, M., et al., *Adipose tissue macrophages in insulin-resistant subjects are associated with collagen VI and fibrosis and demonstrate alternative activation.* Am J Physiol Endocrinol Metab, 2010. **299**(6): p. E1016-27.
42. Lada, A.T., M.C. Willingham, and R.W. St Clair, *Triglyceride depletion in THP-1 cells alters cholesteryl ester physical state and cholesterol efflux.* J Lipid Res, 2002. **43**(4): p. 618-28.
43. Carayol, N., et al., *A dominant function of IKK/NF-kappaB signaling in global lipopolysaccharide-induced gene expression.* J Biol Chem, 2006. **281**(41): p. 31142-51.
44. Wang, S., et al., *Nitric oxide activation of Erk1/2 regulates the stability and translation of mRNA transcripts containing CU-rich elements.* Nucleic Acids Res, 2006. **34**(10): p. 3044-56.
45. Xue, X., et al., *Anti-inflammatory activity in vitro and in vivo of the protein farnesyltransferase inhibitor tipifarnib.* J Pharmacol Exp Ther, 2006. **317**(1): p. 53-60.
46. Taganov, K.D., et al., *NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses.* Proc Natl Acad Sci U S A, 2006. **103**(33): p. 12481-6.
47. O'Connell, R.M., et al., *MicroRNA-155 is induced during the macrophage inflammatory response.* Proc Natl Acad Sci U S A, 2007. **104**(5): p. 1604-9.
48. O'Connell, R.M., et al., *Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder.* J Exp Med, 2008. **205**(3): p. 585-94.
49. Gonsalves, C. and V.K. Kalra, *Endothelin-1-induced macrophage inflammatory protein-1beta expression in monocytic cells involves hypoxia-inducible factor-1alpha and AP-1 and is negatively regulated by microRNA-195.* J Immunol, 2010. **185**(10): p. 6253-64.

50. Shanmugam, N., M.A. Reddy, and R. Natarajan, *Distinct roles of heterogeneous nuclear ribonuclear protein K and microRNA-16 in cyclooxygenase-2 RNA stability induced by S100b, a ligand of the receptor for advanced glycation end products.* J Biol Chem, 2008. **283**(52): p. 36221-33.
51. Schadinger, S.L., et al., *Secretion and antifibrinolytic function of thrombin-activatable fibrinolysis inhibitor from human platelets.* J Thromb Haemost, 2010. **8**(11): p. 2523-9.
52. Garand, M., et al., *Molecular analysis of the human thrombin-activatable fibrinolysis inhibitor gene promoter.* Br J Haematol, 2007. **138**(2): p. 231-44.
53. Wang, E.T., et al., *Alternative isoform regulation in human tissue transcriptomes.* Nature, 2008. **456**(7221): p. 470-6.
54. Wang, M.D., et al., *Differential regulation of ATP binding cassette protein A1 expression and ApoA-I lipidation by Niemann-Pick type C1 in murine hepatocytes and macrophages.* J Biol Chem, 2007. **282**(31): p. 22525-33.
55. Richard, P. and J.L. Manley, *Transcription termination by nuclear RNA polymerases.* Genes Dev, 2009. **23**(11): p. 1247-69.
56. Danckwardt, S., M.W. Hentze, and A.E. Kulozik, *3' end mRNA processing: molecular mechanisms and implications for health and disease.* EMBO J, 2008. **27**(3): p. 482-98.
57. Derti, A., et al., *A quantitative atlas of polyadenylation in five mammals.* Genome Res, 2012. **22**(6): p. 1173-83.
58. Hoque, M., et al., *Analysis of alternative cleavage and polyadenylation by 3' region extraction and deep sequencing.* Nat Methods, 2013. **10**(2): p. 133-9.
59. Smibert, P., et al., *Global patterns of tissue-specific alternative polyadenylation in Drosophila.* Cell Rep, 2012. **1**(3): p. 277-89.
60. Jan, C.H., et al., *Formation, regulation and evolution of Caenorhabditis elegans 3'UTRs.* Nature, 2011. **469**(7328): p. 97-101.
61. Ulitsky, I., et al., *Extensive alternative polyadenylation during zebrafish development.* Genome Res, 2012. **22**(10): p. 2054-66.
62. Millevoi, S. and S. Vagner, *Molecular mechanisms of eukaryotic pre-mRNA 3' end processing regulation.* Nucleic Acids Res, 2010. **38**(9): p. 2757-74.

Chapter 5: Post-transcriptional regulation by microRNA pathway

5.1 Introduction

Haemostasis is achieved by maintenance of the fluidity of blood within the vasculature and protection against excessive blood loss after injury. The respective activities of the coagulation and fibrinolytic cascades are essential to maintain haemostasis. In response to mechanical or chemical injury, the cardiovascular system activates the coagulation cascade to temporarily halt blood flow via formation of the fibrin clot at the site of injury, thus preventing catastrophic blood loss. The fibrinolytic system has evolved to degrade the fibrin clot once the repair to the damaged tissue has ensued. Imbalances between the coagulation and fibrinolytic system can result in a tendency to bleed, as is observed in haemophilia, or to thrombose, as occurs in heart attacks, strokes, and venous thrombosis. A properly functioning vascular system therefore necessitates a delicate balance between coagulation and fibrinolysis.

Thrombin activatable fibrinolysis inhibitor (TAFI) is a plasma zymogen that regulates the balance between coagulation and fibrinolysis, and also mediates molecular connections between coagulation and inflammation [1, 2]. TAFI can be proteolytically activated by thrombin [3], the thrombin-thrombomodulin complex [4], or plasmin [5], resulting in the formation of an enzyme (TAFIa) with basic carboxypeptidase activity. The anti-fibrinolytic function of TAFIa is effected through removal of the carboxyl-terminal lysine residues from partially degraded fibrin that drive the feed-forward loop in the fibrinolytic cascade [1, 6-8]. TAFIa also acts as an anti-inflammatory factor, by inactivating several pro-inflammatory peptides and proteins containing carboxyl-terminal basic residues including bradykinin, the anaphylatoxins C3a and C5a [9], thrombin-cleaved osteopontin [2], bradykinin [10] and plasmin-cleaved chemerin [11]. Significant

variation in plasma TAFI concentrations exists in the human population [12], and several studies have established that elevated plasma TAFI concentrations constitute a risk factor for both arterial and venous thrombotic events [13-20]. It was initially postulated that genetic factors play a major role in influencing the variation in plasma TAFI concentrations. Indeed, many single nucleotide polymorphisms (SNPs) have been identified within *CPB2*, the gene encoding TAFI. The three SNPs that occur in the 3'-untranslated region (3'-UTR) of the *CPB2* mRNA have been shown to affect *CPB2* mRNA stability [22], which may provide the mechanistic basis for lower plasma TAFI levels in individuals harbouring these SNPs. However, subsequent genetic studies have established that only 25% of the total variation in plasma TAFI concentrations can be attributed to polymorphisms in the gene [21] .

In accordance with these findings, we have previously determined a crucial role for the 3'-UTR in mediating both the steady state and regulated changes in *CPB2* mRNA abundance, though modulation of *CPB2* mRNA stability [23]. Additionally, acute phase mediators were found to significantly decrease *CPB2* mRNA abundance in hepatocytes, due to a 2-fold reduction in mRNA half-life [24]. Most recent work in our laboratory has uncovered the presence of one stability and three instability *cis*-elements [36], as well as the ability of *trans*-acting protein factors tristetraprolin (TTP) and HuR to bind to the *CPB2* 3'-UTR (unpublished data) . Numerous studies have established that in addition to protein factors acting on 3'-UTRs of transcripts in post-transcriptional regulation, small non-coding RNA molecules can also assume regulatory functions [25-28]. One example is a class of microRNAs (miRNAs, or miRs), which recognize their target transcripts through Watson-Crick base pairing mechanism (either perfect, or containing mismatches), leading to the recruitment of miRNA induced silencing complex (miRISC),

which in turn recruits CCR4-NOT, resulting in degradation or translational repression of the target mRNA [25, 29-31]. Prior to degradation by the major cytoplasmic 5'-to-3' exonuclease XRN1, mRNAs are first deadenylated and then decapped, which is effected by CCR4-NOT and the decapping complex (DCP1-DCP2), respectively [32]. The process of translational repression by ARE-binding proteins and/or miRISC still remains to be fully elucidated. A recent study proposed that activity of the DEAD-box RNA helicase, eIF4AII, is critical for miRNA-mediated gene silencing and that miRISC inhibits ribosome scanning by recruiting eIF4AII through interaction with the CCR4-NOT complex [33]. Another DEAD-box helicase implicated in this process in human cells is RCK/p54, which has been shown to interact with Ago1 and Ago2 components of miRISC *in vivo* [34]. RCK/p54 induces the formation of P-bodies, which are cytoplasmic foci that house mRNAs destined for translational repression and/or degradation. It is therefore easy to envision a complex regulatory network consisting of ARE-binding proteins, microRNAs and their mRNA targets engaging in molecular mechanisms that govern the regulation of diverse cellular processes.

In order to enhance our understanding of the mechanisms of *CPB2* gene expression regulation, we describe in the current study that *CPB2* mRNA is subject to miRNA-mediated control. This is to our knowledge the first report that describes the functional role of several miRNAs, namely miR-124, miR-143, miR-346, miR-431, miR-506 and miR-708, in modulation of endogenous *CPB2* mRNA and TAFI protein abundance as well as of *CPB2* 3'-UTR reporter activity in HepG2 cells.

5.2 Experimental procedures

5.2.1. Mammalian culture and transfections

Cells were maintained in a humidified incubator at 37°C under a 95% room air/5% CO_2 atmosphere. HepG2 cells and THP-1 monocytes were cultured as previously described [36]. THP-1 monocytes were differentiated into macrophages with phorbol-12-myristate-13-acetate (PMA), as previously described [37]. Transfections of HepG2 cells with the luciferase reporters and miR mimics were performed with Megatran (OriGene), as per the manufacturer's protocol. MiRNA inhibitors (anti-miRs) were purchased from Qiagen in the form of locked nucleic acids (LNAs), and transfection of anti-miRs were performed with Attractene (Qiagen), as per manufacturer's protocol. The cells were incubated with transfection complexes for 48 hours. For endogenous *CPB2* mRNA quantitation, RNA was extracted using Trizol reagent (Invitrogen), followed by genomic DNA removal using DNaseI (Promega). *CPB2* mRNA was quantitated using qRT-PCR as previously described [36], using primer and probe set specific for human TAFI (forward primer: 5'-GGA TTT CTATGT TAT GCC GG-3'; reverse primer: 5'-GAT TGT TCG CAT AGA AAG AAC-3'; probe: 5'-/56-FAM/CCA CAT TCG /ZEN/ATT CTT TTT CCATGA GTA GTC ATA ACC GTC C/3IABkFQ/-3', where 6-FAM is the fluorescent dye and ZEN and Iowa Black FQ [3IaBkFQ] are quenchers), and human GAPDH (forward primer: 5'-TGT AGT TGA GGT CAATGA AGG G-3'; reverse primer: 5'-ACATCG CTC AGA CAC CAT G-3'; probe: 5'-/5HEX/AAG GTC GGA /ZEN/GTC AAC GGATTT GGT C/3IABkFQ/-3', where HEX is the fluorescent dye and ZEN and Iowa Black FQ [3IaBkFQ] are quenchers)for normalization. For studies on endogenous TAFI protein, after transfection of miRNA mimics, the cells were incubated in serum-deficient media, and the conditioned media was collected 48 hour later for

immunoblotting. In parallel, cell lysates were collected in lysis buffer (50 mM Tris HCl pH 7.4, 1% v/v NP-40, 0.25% w/v sodium deoxycholate, 150 mM NaCl, 1mM EDTA) and subjected to immunoblotting. For western blot analyses, experiments were performed using SDS-PAGE on 10% polyacrylamide gels under non-reducing conditions, and blotted onto PVDF membrane. The membrane with conditioned media samples was probed with sheep anti-human TAFI antibody (Affinity biologicals) in 3% (w/v) non-fat milk in 1× NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, 0.5% Triton X-100) while the membrane with the lysate samples was probed with mouse anti human-β-actin antibody (Sigma Aldrich) in 1 x TBST (0.15M NaCl, 20mM Tris-HCl, 0.1% Tween-20, pH 7.4). Immunoreactive bands were visualised using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and a FluorChem Q Gel Imaging System (Alpha Innotech).

5.2.2 Synthesis of microRNA mimics

Oligonucleotide primers encoding each strand of the miRNA duplex sequences and ending with the T7 priming sequence were obtained from Integrated DNA Technologies. The primers for the miRNAs used in this study were as follows:

1. miR-124 guide: 5’-ATC AAG GTC CGC TGT GAA CAC GTT CCT ATA GTG AGT CGT ATT A-3’
2. miR-124 passenger: 5’-GGC ATT CAC CGC GTG CCT TAT TCC TAT AGT GAG TCG TAT TA -3’
3. miR-133a guide: 5’- CAG CTG GTT GAA GGG GAC CAA ACCTAT AGT GAGTCG TAT TA -3’
4. miR-143 guide: 5’- GAG CTA CAGTGC TTC ATC TCA CCT ATA GTG AGT CGT ATT A -3’
5. miR-346 guide: 5’- ACA GGC AGG CAT GCG GGC AGA CAC CTATAGTGA GTC GTATTA -3’
6. miR-346 passenger 5’ CTG CAG GCC CAG CCC CTG CCT CCCTAT AGT GAGTCG TAT TA -3’
7. miR-431 guide: 5’- TGC ATG ACG GCCTGC AAG ACA CCT ATA GTG AGT CGT ATT A -3’
8. miR-431 passenger: 5’- AGA AGC CCT GCA AGA CGA CCT GCCTAT AGT GAGTCG TAT TA -3’

9. miR-506 guide: 5'- TCT ACT CAG AAG GGT GCCTTA CCT ATA GTG AGT CGT ATT A -3'

10. miR-708 guide: 5'- CTA GAA GCT CAC AGT CTA GTT GCCTAT AGT GAGTCG TAT TA -3'

11. T7 primer sequence: 5'-TAA TAC GAC TCA CTA TAG G-3'

To synthesize the miRNA duplexes, first the template was made by annealing equimolar amounts of the miRNA primer and the T7 primer. *In vitro* transcription was then carried out overnight, using T7 High Yield RNA synthesis kit (New England Biolabs). The samples were treated with DNase I (Promega) to remove the DNA template and miRNAs were extracted with phenol:chloroform: isoamyl alcohol (25:24:1) (ACP Chemicals Inc.). RNA concentrations were then measured using the Nanodrop 2000 Spectrophotometer (Thermo Fischer Scientific) and the equimolar amounts of each guide strand was annealed to the corresponding passenger strand to form the miRNA duplex. In some cases, only the guide strand sequence was available from the miRNA database, in which case this was the only strand synthesized and used for transfection into HepG2 cells.

5.2.3 RNA-binding protein purification and identification (RaPID)

The RaPID procedure relies on the use of two main constructs as previously described [38]. pN-RFPx24 and pUG34-MS2-CP-GFP-SBP, which were the kind gift from Dr. Jeffrey Gerst (Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel). Since pUG34-MS2-CP-GFP-SBP is a yeast plasmid, we first removed the sequence encoding the fusion protein CP-GFP-SBP by digestion with *Xba*I, and then inserted it into pcDNA-4B plasmid pre-digested with *Xba*I. The *CPB2* 3' flanking region was inserted into pN-RFPx24 plasmid by excising this segment from βG-*CPB2*/3'UTR (see above) with *Xho*I and *Sal*I , making the ends blunt with T4 DNA

polymerase and inserting into pN-RFPx24 digested with *BamH*I and blunted. HepG2 cells were transfected in 100-mm dishes with pcDNA4B-CP-GFP-SBP and pN-RFPx24-*CPB2*/3'UTR simultaneously using Lipofectamine 2000 reagent for 48 hours. Collection and lysis of cells was carried out as previously described , with a modification for cross-linking, which was done in phosphate-buffered saline (PBS) containing 0.5% (v/v) formaldehyde for 10 min at 24°C with slow shaking. The pulldown assay was performed as previously described [38]. For western blot analyses, experiments were performed using SDS-PAGE on 4-15% polyacrylamide gradient gels under non-reducing conditions. The blots were incubated with HRP-linked polyclonal goat anti-human Ago2, which was a kind gift of Dr. Sirinart Ananvoranich (University of Windsor, Windsor, Canada), in blocking buffer containing 3% (w/v) non-fat milk in 1× NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, 0.5% Triton X-100) and incubated at 4°C overnight. Immunoreactive bands were visualised using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and a FluorChem Q Gel Imaging System (Alpha Innotech).

5.2.4 Luciferase reporter plasmid construction and luciferase assay

The pC7β-globin vector containing the *CPB2* 3'-flanking region was constructed first. A segment of the *CPB2* 3'-flanking region beginning at the stop codon and extending for 1 kb downstream was amplified from human genomic DNA. The segment was inserted into pC7βG digested with *Pac*I (just downstream of the translation termination cassette) and *Sal*I. In utilizing this downstream *Sal*I site in pC7βG, the SV40 polyA signal was removed prior to insertion of the genomic DNA segment. *CPB2*/3'-UTR-luc construct was made by inserting the luciferase gene digested from pGL3 basic

vector with *Nhe*I and *Xba*I. The ends were made blunt with T4 DNA polymerase and the blunt-end ligation was achieved by digestion of pC7β-globin-*CPB2/*3'-UTR vector with *Xho*I and T4 DNA polymerase blunting. The TTP binding site in the 3'-flanking region was mutated as previously described [36], for generation of the ΔTTP/3'UTR-luc construct. T+ 1583 site directed mutagenesis was carried out using QuickChange II Site-directed mutagenesis kit (Agilent technologies), and mutagenic primers (forward primer: 5'- GAT CAG CGT GAG ATG ATC ATT GAT TAA ACT TGC TTG AGATG -3'; reverse primer: 5'- CAT CTC AAG CAA GTT TAATCA ATG ATC ATC TCA CGCTGA TC -3'), in the context of the *CPB2*/3'UTR-luc vector. The constructs were transfected into HepG2 cells using Megatran (OriGene), along with pRl-tk plasmid encoding Renilla luciferase under the control of thymidine kinase promoter. After 48 hours, the cell lysates were collected in Passive Lysis Buffer (Promega). Reporter activities were measured using the dual luciferase reporter system. The firefly luciferase activity was measured by adding the firefly substrate D-luciferin (Sigma Aldrich) in firefly buffer (20mM Tricine, 10mM MgSO4, 5mM DTT, 250μM Coenzyme A, 250μM ATP). Renilla luciferase was measured by adding renilla substrate coelentrazine (Sigma Aldrich) in the renilla buffer (100mM KPO_4 buffer pH 7, 500mM NaCl, 1mM EDTA, 0.002% BSA). The luciferase signals were measured with the Turner BioSystems Luminometer 20/20n (Promega).

5.3 Results

5.3.1 Endogenous expression levels of several miRNAs are different in HepG2 cells, THP-1 monocytes and THP-1 macrophages

As an initial step, we performed bioinformatic analysis of *CPB2* 3'-UTR using miRNA prediction software (www.microRNA.org and www.miRBase.org) and identified several miRNAs with high probability scores (mirSVR score from -0.5193 to -1.1520) and that were conserved across species (Figure 5.1). In order to examine the potential involvement of the microRNA pathway in *CPB2* gene expression regulation, we performed a recently described technique that allows for identification for RNA and protein factors present in ribonucleoprotein complexes, called RNA-binding protein purification and identification (RaPID) [38]. Previously in our laboratory, we used the RaPID technique successfully to identify two protein factors that associate with *CPB2* 3'-UTR, tristetraprolin (TTP) and HuR (unpublished data). In the present investigation, we sought to determine whether protein factors present in the RISC complex (such as Ago2) associate with the 3'-UTR of *CPB2* transcript. We generated the *CPB2* 3'-UTR 'bait' construct by inserting the 3'-flanking region downstream of the aptamer sequences (MS2) repeated 24 times, that specifically and with high affinity associate with bacteriophage coat protein, which is encoded by the 'hunter' construct. In addition, the 'hunter' also contains streptavidin binding protein for affinity purification step using streptavidin beads. Both of the constructs were then transfected into HepG2 cells. Therefore, through specific interaction between streptavidin-binding protein and streptavidin beads, we were able to pull down the protein factors that associate with the *CPB2* 3'-UTR. This material was then probed with the anti-Ago2 antibody using immunoblotting. Due to the presence of multiple immunoreactive bands present in input and eluate lanes, we were not able to

conclusively establish that Ago2 specifically associates with the *CPB2* 3'-UTR; nevertheless, a band of the size corresponding to Ago2 appears more prominent in the lane containing lysate from cells expressing the *CPB2/*3'UTR-contacting bait plasmid (Fig.5.2), suggesting that Ago2 may be associating with *CPB2* 3'-UTR *in vitro* in HepG2 cells. This prompted us to investigate the potential roles for various candidate miRNAs, namely miR-124, miR-143, miR-506 and miR-708 in mediating *CPB2* gene expression regulation.

As the expression level of many miRNAs is deregulated in cancer [39], and HepG2 cells and THP-1 monocytes are immortalized cell lines (human hepatocellular carcinoma and human monocytoid leukemia, respectively), we first examined the expression levels of individual miRNAs in these cells lines. We detected the expression of miR-124, miR-143, and miR-506 in both cells lines, while miR-708 was only detectable in HepG2 cells (Figure 5.3). However, there was a dramatic difference in their levels of expression between the two cell lines. Namely, miR-124 is expressed at a 500-fold higher level in THP-1 monocytes compared to HepG2 cells, while miR-143 expression was found to be 15-fold higher in THP-1 monocytes than in HepG2 cells. The expression level of miR-506 was the same for these two cell lines, while miR-708 was expressed endogenously only by HepG2 cells. Moreover, since the components of the regulatory cellular machineries can differ depending on the state of differentiation of a particular cell [40-42], we investigated the possibility that the miRNA expression pattern differs between THP-1 monocytes and macrophages derived from differentiation of these cells. Indeed, miR-124 expression level was even higher in THP-1 macrophages compared to THP-1 monocytes and HepG2 cells (2-fold and 1000-fold, respectively). The same was observed for miR-143, which was expressed at a 30-fold higher level when

THP-1 cells were differentiated, and 500-fold higher compared to HepG2 cells (Figure 3). Additionally, while the expression of miR-506 is similar in HepG2 cells and THP-1 monocytes, we observed a 15-fold higher expression in THP-1 macrophages. Differentiation of THP-1 cells however did not induce expression of miR-708, as it was undetectable in THP-1 macrophages.

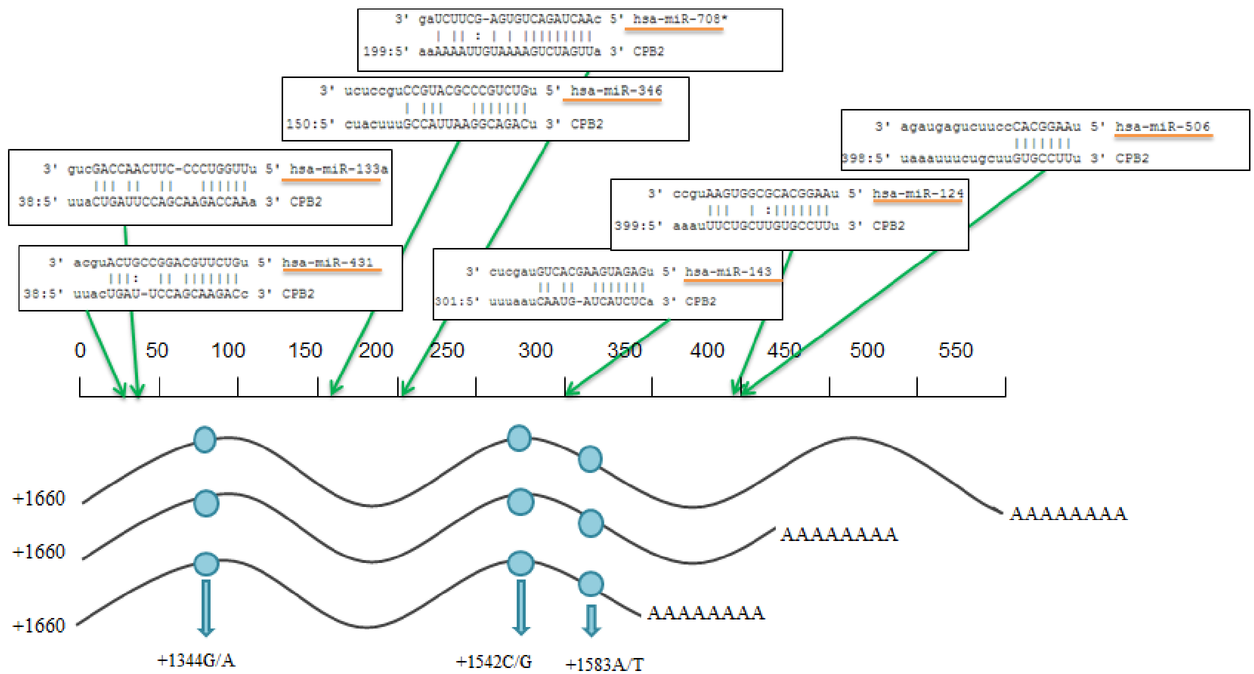

Figure 5.1 Bioinformatic analysis of target predictions within *CPB2* 3'-UTR. Bioinformatic analysis was performed using microRNA database (www.microRNA.org) and miRBase (www.miRBase.org). The alignments shown here were obtained from the former.

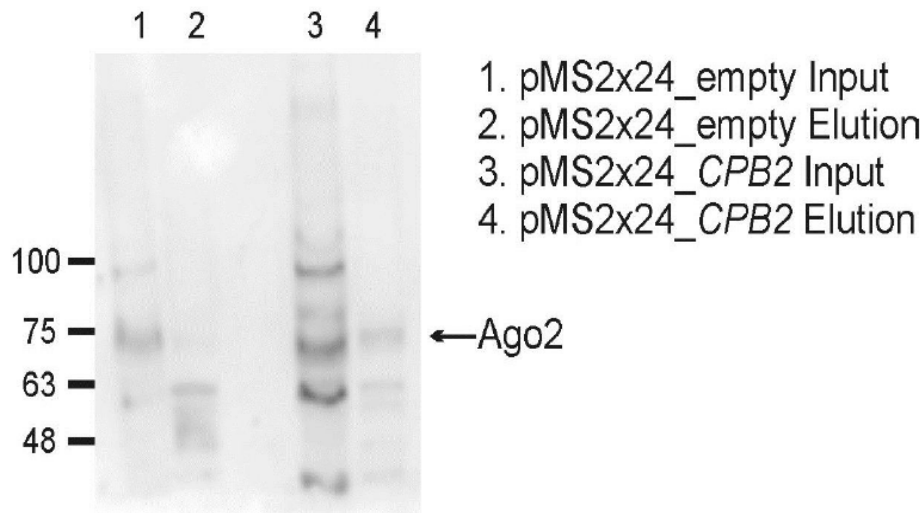

Figure 5.2 Identification of Ago2 as a trans-acting factors binding to the *CPB2* 3'-UTR. We adapted an aptamer-based mRNA affinity purification technique for the identification of RNA and protein factors present in ribonucleoprotein complexes. HepG2 cells were co-transfected with the 'bait' construct encoding the aptamer repeats and the *CPB2* 3'-UTR and the 'hunter' construct encoding the aptamer-binding protein and lysates were subjected to affinity chromatography. The input, elution and wash fractions from the chromatography were subjected to Western blot analysis using an anti-Ago2 antibody Control experiments were performed using a 'bait' construct lacking the *CPB2* 3'-UTR and the elution fraction was also included on the Western blots (No 3'-UTR).

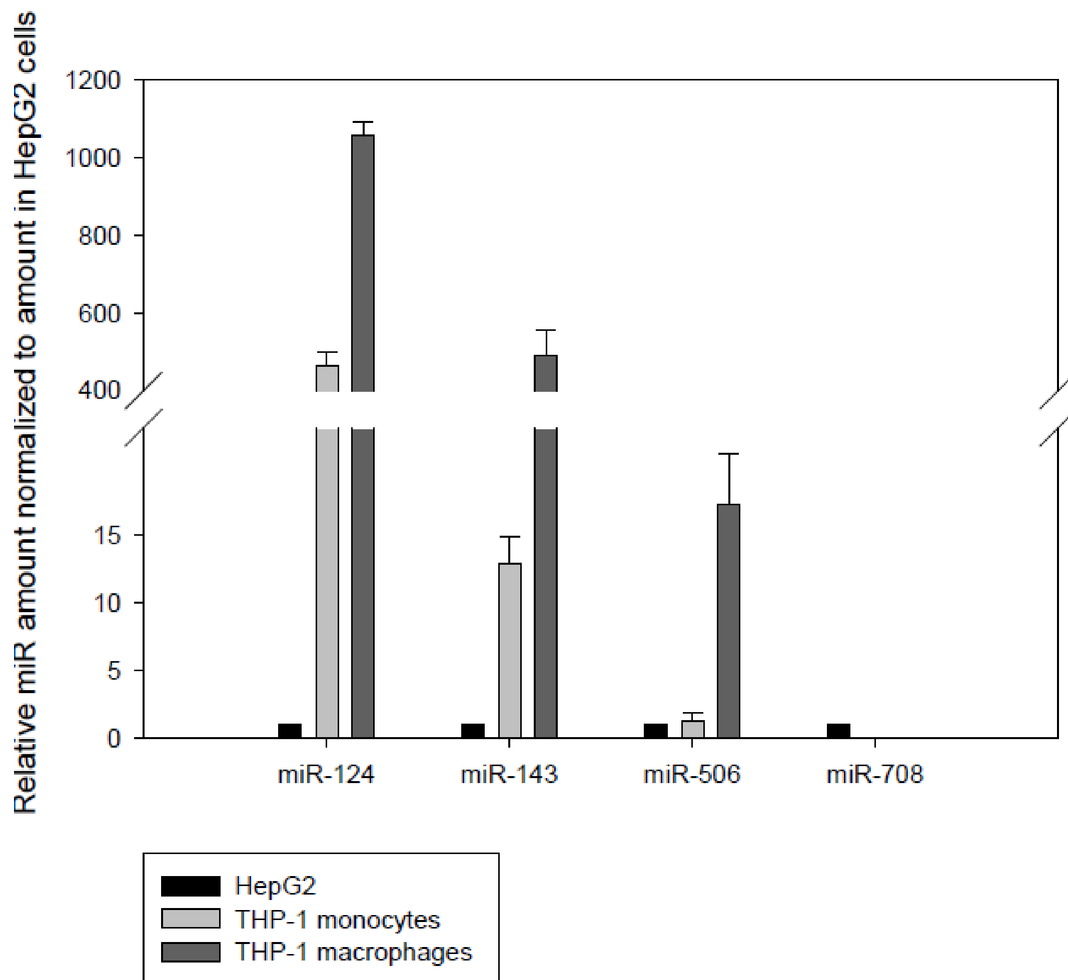

Figure 5.3 Quantitation of miRNAs endogenously produced by HepG2 cells, THP-1 monocytes and THP-1 macrophages. RNA was extracted from the respective cell types and subjected to reverse transcription to generate cDNA. Individual miRs (miR-124, miR-143, miR-506 and miR-708) were detected by using miR-specific primers and miScript SYBR Green RT-PCR kit. In parallel, reactions containing primers specific for β-actin were carried out for normalization. The relative abundance of each miR was calculated using the ΔCt method.

5.3.2 Endogenous *CPB2* mRNA and TAFI protein levels are modulated by microRNAs

To determine whether *CPB2* mRNA is a target for these miRNAs *in vitro*, we investigated the effect of their overexpression in HepG2 cells on the levels of endogenously expressed *CPB2* transcript and TAFI protein. Here we also included three additional miRNA candidates, miR-133a, miR-346 and miR-431, which have also been predicted by the database, albeit with lower probability scores. Oligonucleotide mimics of miR-124, miR-133a, miR-143, miR-346, miR-431, miR-506, and miR-708 were introduced into HepG2 cells and total RNA was extracted for quantitation of *CBP2* mRNA with qRT-PCR. We observed that miR-124, miR-506, and miR-708 significantly reduced *CPB2* mRNA levels, while miR-133a, miR-143, miR-431 and miR-346 had no significant effect (Figure 5.4). Additionally, these effects were reversed in the presence of specific anti-miRs that antagonized the action of the corresponding miRNAs (Figure 5.5). The anti-miRs effectively reversed the decrease in *CPB2* mRNA abundance, both when they were transfected with miRNA mimics, and on their own (in the endogenous context). We then examined whether these changes in mRNA abundance are also reflected in the level of TAFI protein produced by these cells. Indeed, TAFI protein abundance was significantly reduced when we overexpressed miR-124, miR-506 and miR-708 (Figure 5.6). Interestingly, miR-346 overexpression did not significantly affect *CPB2* mRNA levels, but resulted in a decrease in TAFI protein. It appears that miR-346 possesses some capacity to modulate *CPB2* gene expression, although its role may not be as prominent as those of miR-124, miR-506 and miR-708.

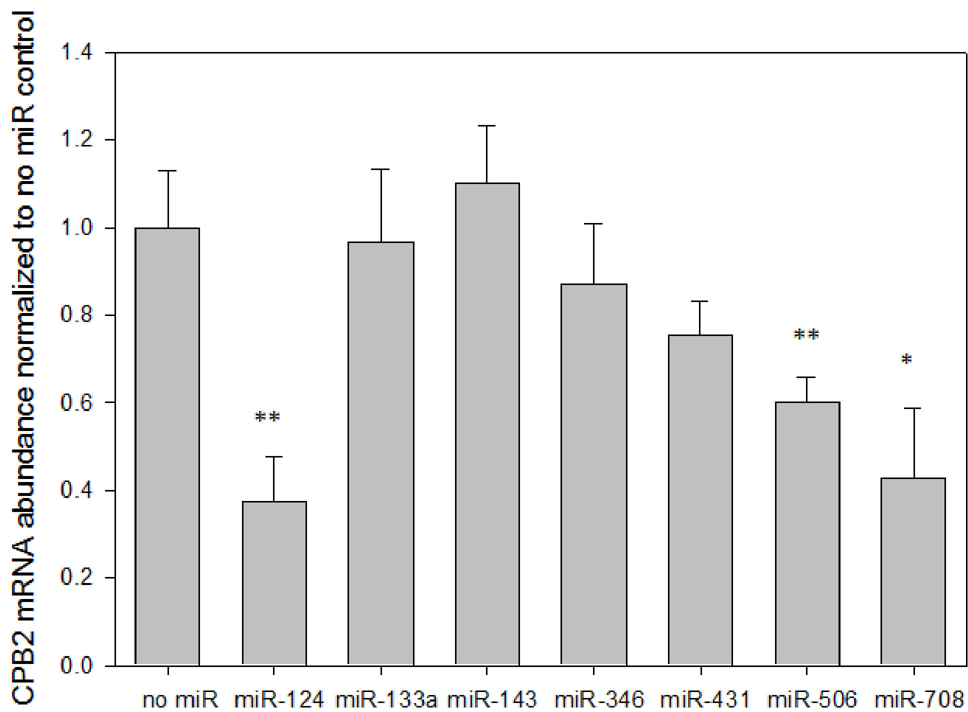

Figure 5.4 miR-124, miR-431, miR-506 and miR-708 modulate abundance of endogenous *CPB2* mRNA. HepG2 cells were transfected with indicated miR mimics for 48 hours, followed by isolation of total RNA and DNase I digestion. Endogenous *CPB2* mRNA was quantitated with real-time qRT-PCR using Taq-based chemistry in a multiplex reaction containing primer and probe sets specific for human TAFI and GAPDH. The relative abundance of TAFI mRNA for each sample was then normalized to negative control. The data shown are the mean ± s.e.m. of five independent experiments. *: $p < 0.05$; ** $p < 0.001$ versus no miR control by Student's t-test.

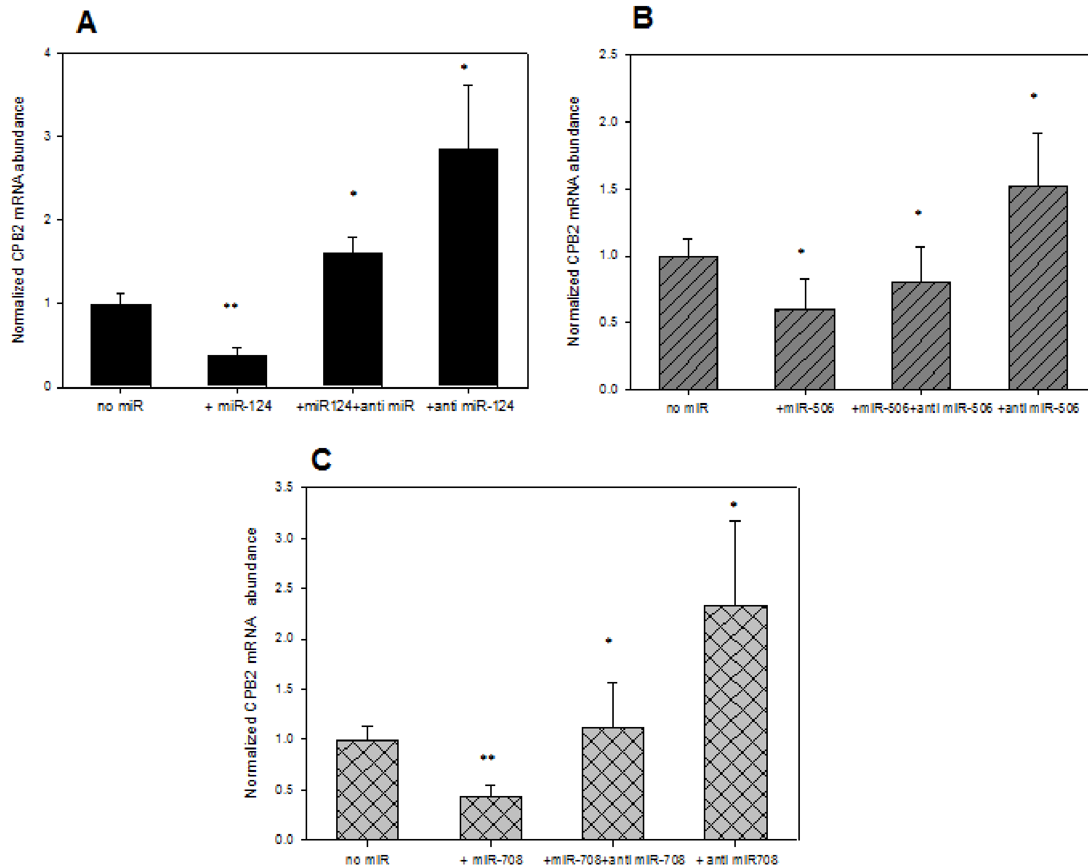

Figure 5.5 miR-mediated decrease in *CPB2* mRNA abundance is reversed by specific anti-miRs. HepG2 cells were grown in 12-well plates and transfected with indicated miR-mimics and specific miR inhibitors (anti-miRs), where appropriate, for 48 hours, followed by isolation of total RNA and DNase I digestion. Endogenous *CPB2* mRNA was quantitated with real-time qRT-PCR using Taq-based chemistry in a multiplex reaction containing primer and probe sets specific for human TAFI and GAPDH. The relative abundance of TAFI mRNA for each sample was then normalized to negative control. The data represents the average of two independent experiments. *: $p < 0.05$; ** $p < 0.001$ versus no miR control by Student's t-test.

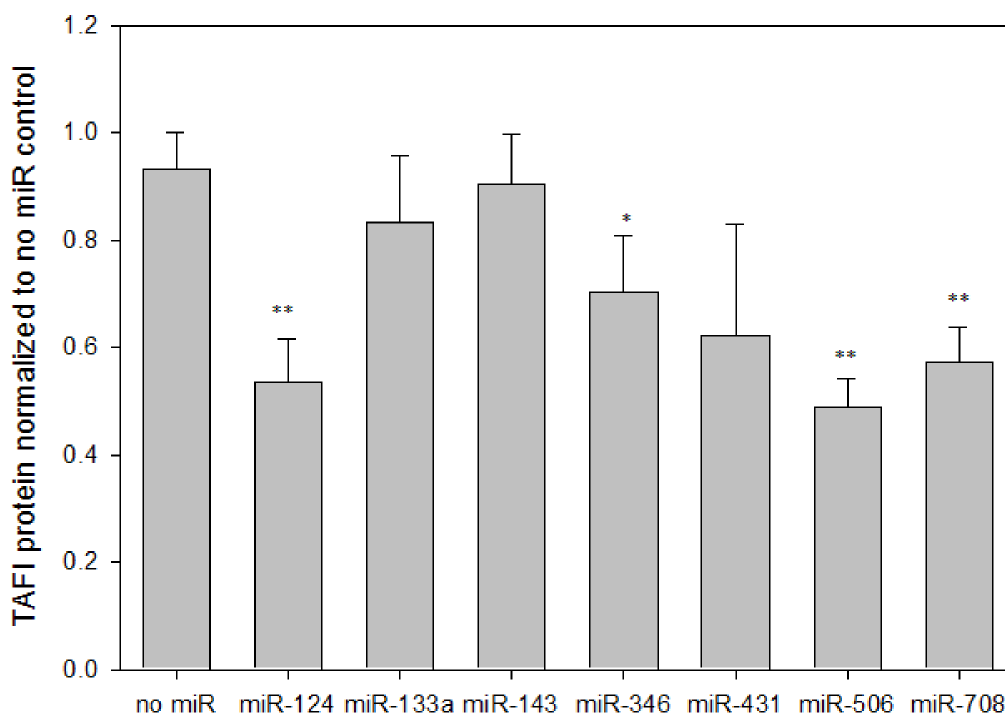

Figure 5.6 miR-124, miR-346, miR-506 and miR-708 modulate TAFI protein levels in HepG2 cells. HepG2 cells were grown in 6-well plates and transfected with indicated miR mimics. Conditioned media was collected at various time points and the amount of TAFI protein was determined with immunoblotting using antibody specific for human TAFI. TAFI amounts in each sample were corrected to their corresponding total cellular protein content, by Western blotting of lysates with β-actin antibody and expressed relative to the TAFI amount present in the no miR sample. The data represents the average of two independent experiments. *: $p < 0.05$; ** $p < 0.005$ versus no miR control by Student's t-test.

5.3.4 *CPB2* 3'-UTR is a functional target of candidate microRNAs

To determine whether *CPB2* mRNA is a functional target of miR-124, miR-346, miR-431, miR-506 and miR-708, 1 kilobase of *CPB2* 3'-flanking region was cloned into a luciferase reporter vector (*CPB2*/3'-UTR-luc). Additionally, a reporter vector was also constructed which lacked the *CBP2* 3'-UTR (NO 3'-UTR-luc), to ensure that any changes in reporter activity are attributable to changes mediated by the 3'-UTR. The data showed a significant decrease in luciferase activity in the presence of all candidate miRNAs compared to the negative control, while there was no change in the activity of the 3'UTR-less reporter (Figure 5.7).

5.3.5 miR-124 target site overlaps with TTP binding site within *CPB2* 3'-UTR

Bioinformatic analysis of the predicted miR-124 target sequence revealed that it overlaps proximally with the binding site of TTP, an mRNA destabilizing factor that we have previously characterized as an important regulator of *CPB2* mRNA stability and abundance [36]. In order to investigate whether this is the case, we introduced a TTP binding site mutation (AATAAATTT→ AAGAAAGGG) in the context of *CPB2*/3'-UTR luciferase reporter vector. In this mutation, the two most distal nucleotides (TT→GG) represent the change that would affect the most proximal two nucleotides of the predicted miR-124 target sequence. We transfected the wild-type *CPB2*/3'-UTR-luc (in which the binding site of TTP is intact) and the mutant (ΔTTP/3'-UTR-luc) luciferase constructs into HepG2 cell and measured the reporter activity in the presence of miR-124 mimic. The reporter activity of *CPB2*/3'-UTR-luc was decreased in the presence of miR-124, whereas the ΔTTP/3'-UTR-luc reporter was refractory to these changes (Figure 5.8A).

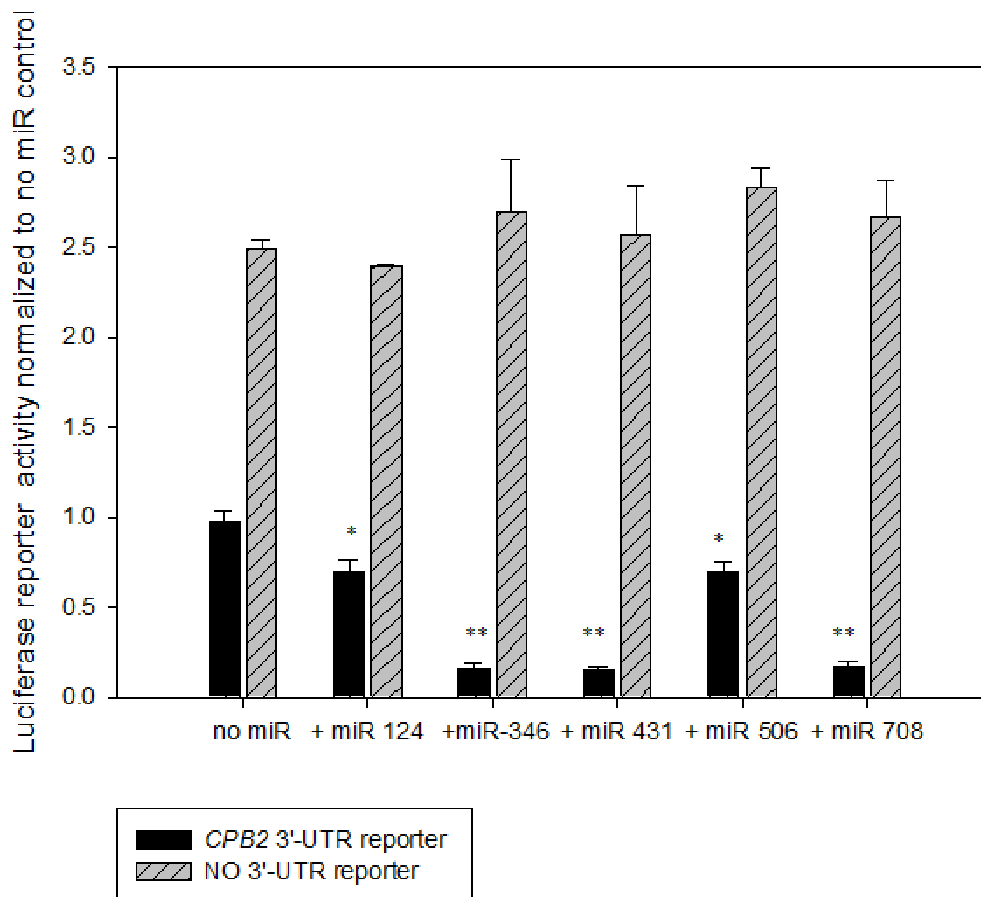

Figure 5.7 ***CPB2*** **3'-UTR is a functional target of candidate miRs.**
HepG2 cells were transfected with luciferase reporter constructs harbouring *CPB2* 3'-flanking region (*CPB2*/3'-UTR-luc), or the 3'-UTR-less constructs (NO3'-UTR-luc), together with the indicated miR mimics for 48 hours. Reporter activity was measured with dual luciferase reporter system, by normalizing firefly luciferase signal with renilla luciferase signal, which were then normalized to no miR control. The data represents the average of two independent experiments. *: $p < 0.05$; ** $p < 0.001$ versus no miR control by Student's t-test.

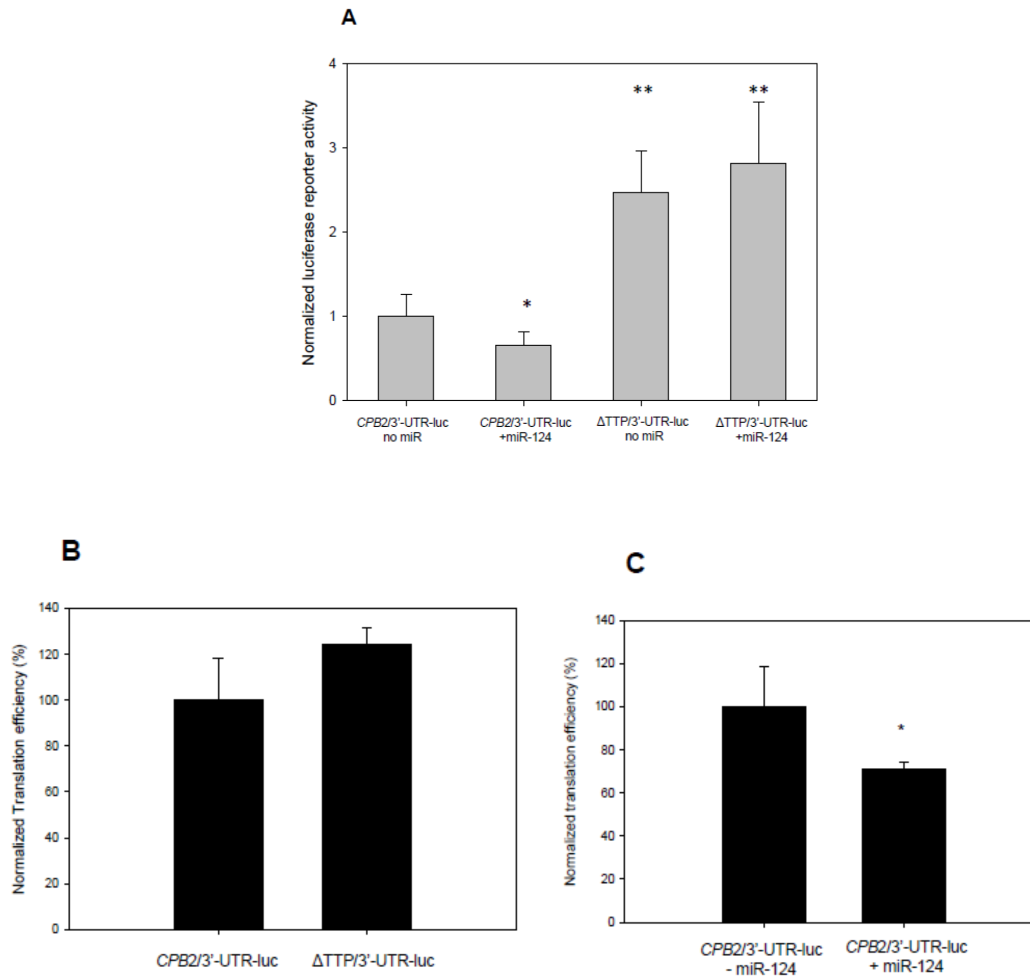

Figure 5.8 miR-124 decreases translation efficiency of *CPB2* transcript and requires intact TTP binding site for efficient targeting. HepG2 cells were transfected with luciferase reporter constructs harbouring *CPB2* 3'-flanking region and either intact TTP binding site (*CPB2*/3'-UTR-luc), or containing the TTP binding site mutation (ΔTTP/3'-UTR-luc) , together with miR-124 mimic, where appropriate, 48 hours. A) Reporter activity was measured with dual luciferase reporter system, by normalizing firefly luciferase signal with renilla luciferase signal, which were then normalized to no miR control. The data represents the average of two independent experiments. *: $p < 0.02$; ** $p < 0.001$ versus no miR *CPB2*/3'-UTR-luc control by Student's t-test. B) For translation efficiency determination, RNA was extracted in parallel to collection of lysates for luciferase assay and the amount of reporter transcript was determined with qRT-PCR in a multiplex reaction, using primer and probe sets specific for rabbit β-globin and human GAPDH sequences. The normalized luciferase signal was divided by the abundance of the reporter transcript, thus indicating reporter activity per unit mRNA. The data represent the average of two independent experiments. *: $p < 0.01$ versus no miR *CPB2*/3'-UTR-luc control by Student's t-test.

Of note, the reporter activity of ΔTTP/3'-UTR-luc in the absence of miR-124 mimic was significantly higher than that of the wild-type *CPB2*/3'-UTR-luc, in keeping with the role of TTP as an mRNA destabilizing factor.

Next we sought to examine whether miR-124 affects translation efficiency. For this purpose, we determined luciferase reporter activity per unit *CPB2* mRNA in the presence of miR-124 mimic. The results showed that in fact, translation efficiency is significantly reduced when miR-124 is overexpressed, compared to the negative control (Figure 8C). We also examined translation efficiency of the transcript lacking the functional TTP binding site and found no difference compared to the wild-type control (Figure 8B). Therefore, TTP does not seem to modulate translation efficiency of *CPB2* mRNA, whereas miR-124 appears to play a role in this process.

5.3.6 T+1583A SNP allele is selectively targeted by miR-143

That inter-individual plasma TAFI variability arises due to genetic factors has been addressed in the past by genetic studies [21, 35]. Gene polymorphisms that occur in both the 5'- and the 3'-flanking region of *CPB2* gene are in strong linkage disequilibrium with each other, and with the previously described Ala147Thr polymorphism [22]. Of the SNPs that occur in the 3'-UTR of *CPB2* mRNA, the T+ 1583A has been associated with lower plasma TAFI levels in both homozygous and heterozygous individuals [35]. Here we investigated a potential mechanistic basis for the TAFI-lowering effect of this SNP. Namely, miR-143 was predicted to target the sequence encompassing the T+ 1583A SNP. In fact, miR-143 seed sequence is complementary to the T allele, and is predicted to perfectly base-pair with the allele bearing T at this position.

In our initial studies on the effect of miRNAs on endogenous *CPB2* mRNA and protein levels, we observed no change in mRNA and TAFI protein levels in the presence

of miR-143 mimic (Figure 5.6). This prompted us to investigate the genotype of HepG2 cells. We synthesized cDNA from RNA extracted from HepG2 cells and performed sequencing analysis. Indeed, HepG2 cells harbour the A allele at this position. This may explain why miR-143 mimic caused no change in *CPB2* mRNA levels. We then sought to determine the genotype of the luciferase reporter construct, as this construct was also refractory to miR-143 downregulation. As expected, the 3'-flanking region within the reporter construct also contains the A allele at position +1583. We then performed site directed mutagenesis to generate the A→T mutant in the context of the luciferase reporter construct, that now harboured the T allele at position +1583. We predicted that by doing so, the luciferase reporter will be susceptible to miR-143 downregulatory effects. Indeed, we observed a 40% decrease in reporter activity for the T+ 1583 mutant in the presence of miR-143 mimic, whereas the reporter bearing the A allele at this position was refractory to miR-143- mediated downregulation in activity (Figure 5.9). These results may, at least in part, explain the mechanistic basis for plasma TAFI-lowering effect of the T+ 1583A SNP.

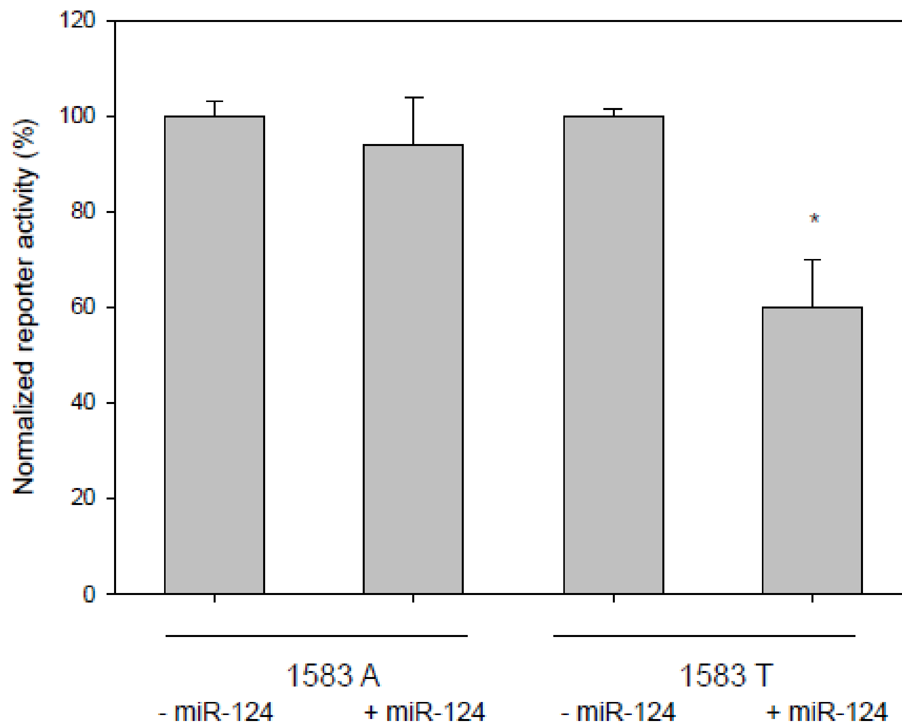

Figure 5.9 T+1583 SNP is a functional target of miR-143
HepG2 cells were transfected with luciferase reporter constructs harbouring *CPB2* 3'-flanking region and either harbouring the A allele at position +1583 (1583 A) or the T allele (1583 T), together with miR-143 mimic for 48 hours. Reporter activity was measured with dual luciferase reporter system, by normalizing firefly luciferase signal with renilla luciferase signal, which were then normalized to no miR control. The data represent the average of three independent experiments. *: $p < 0.01$ versus no miR 1583 T control by Student's t-test.

5.4 Discussion

Plasma TAFI levels vary substantially in the human population, and elevated plasma TAFI concentrations have been associated with various thrombotic and atherothrombotic disorders. Genetic studies have established that only about a quarter of this variability can be attributed to genetic factors, underscoring the potential prominent role for the gene regulatory component. Nonetheless, several SNPs that occur throughout the *CPB2* gene have been strongly associated with plasma TAFI concentrations and contributed a significant component of plasma TAFI variability [21, 35]. Due to the relatively long length (390-549 nucleotides) of the 3'-UTR, it likely serves as a crucial nexus for regulation of *CPB2* gene expression. Numerous studies on post-transcriptional regulation uncovered novel mechanisms that orchestrate the events at the 3'-UTR of protein coding mRNAs. It is becoming increasingly apparent that there is significant crosstalk between these mechanisms [43]. For example, microRNAs and AU-rich element RNA (ARE)-binding proteins that possess binding sites within shared mRNAs often act simultaneously or in concert to exert their downregulatory effects. We have recently uncovered an important role for post-transcriptional regulation in mediating the effects of inflammatory mediators on *CPB2* mRNA and TAFI protein abundance, in which TTP seems to play a crucial role through modulation of mRNA stability (unpublished data). The first line of evidence that *CPB2* 3'-UTR might be susceptible to microRNA-mediated control arose from our RaPID experiments, in which we were able to detect Ago2 in the eluate fraction of proteins that associated with the 3'-UTR of *CPB2* (Figure 2). The binding of Ago2 was *CPB*2 3'-UTR-specific, as the Ago2 immunoreactive band was absent from the elution faction for the construct lacking the 3'-UTR of *CPB2*. In the

current study, we sought to further our understanding of *CPB2* gene expression regulation at the post-transcriptional level mediated by the microRNA pathway.

Initial bioinformatic analysis revealed that several miRNAs may potentially target *CPB2* 3'-UTR, and we selected those with high scores and conservation across species to begin our investigation (Figure 5.1). We first examined the levels of endogenous miR-124, miR-143, miR-506 and miR-708 expressed by HepG2 cells, THP-1 monocytes and THP-1-macrophages. Although the expression of *CPB2* in the liver is likely the primary contributor to the plasma pool of TAFI, extra-hepatic sites of TAFI expression have recently been described by our group and others [37, 44, 45]. We have, for the first time, reported the expression of *CPB2* in THP-1 monocytes and macrophages derived from differentiation of monocytes, as well as TAFI protein production by THP-1 macrophages, but not THP-1 monocytes [37]. Here we examined whether the expression level of individual miRNAs varies in different cell types that *CPB2* is expressed in (Figure 5.3). Interestingly, we found that the level of expression of miR-124 was 500-fold higher in THP-1 monocytes, and 1000-fold higher in THP-1 macrophages compared to HepG2 cells. Additionally, miR-143 also displayed a prominent induction upon differentiation of THP-1 monocytes from 15-fold to 500-fold higher than in HepG2 cells. Curiously, quantitation of endogenous levels of *CPB2* mRNA revealed that the number of transcripts present in THP-1 monocytes is approximately 1250-fold lower compared to HepG2 cells, and even lower (by approximately 5000-fold) in THP-1 macrophages [37]. It is tempting to speculate that *CPB2* mRNA may be subjected to a more rigorous control by microRNAs in THP-1 monocytes and macrophages and that this control may, at least in part be responsible for the lower abundance of the transcript in these cell types. Of note, miR-708 was detected in HepG2 cells, unlike THP-1 monocytes and macrophages, which

may imply the role of miR-708 in tissue-specific control of *CPB2* gene expression regulation.

In order to investigate the potential of candidate miRNAs to modulate *CPB2* gene expression *in vitro*, we synthesized oligonucleotide mimics corresponding to miR-124, miR-143, miR-506 and miR-708, and also included miR-133a, miR-346 and miR-431, which were also predicted by the database, but had lower probability scores. When we introduced these miRNA mimics into HepG2 cells, we observed a decrease in *CPB2* mRNA abundance in the presence of miR-124, miR-506 and miR-708, while miR-133a, miR-143 and miR-346 did not affect the transcript levels (Figure 5.4). We also confirmed that these effects are miRNA specific in case of miR-124, miR-506 and miR-708 by introduction of specific anti-miRs. MiR antagonism relieved the depression of *CPB2* mRNA levels, both on their own (when they are expected to antagonize the endogenous miRNA only) and when administered in combination with the corresponding miRNA (when they are expected to antagonize both the mimic and the endogenous miRNA) (Figure 5.5). In fact, this effect was the highest in the presence of the anti-miR alone, whereas it occurred to a slightly lower extent when the corresponding miRNA was administered simultaneously. The reason for this difference may arise due to the depletion of anti-miR molecules in the presence of both the mimic and the endogenous miRNA. Importantly, the expression level was increased in the presence of anti-miRs alone compared to the basal *CPB2* abundance, suggesting that the antagonism of specific endogenous miRNAs leads to an increase in abundance of endogenous *CPB2* mRNA.

In order to confirm that *CPB2* 3'-UTR is a direct functional target of these miRNAs, we performed luciferase reporter assay. For this purpose, we constructed luciferase reporter constructs by inserting a 1 kb of the *CPB2* 3'-flanking region, thus

anticipating that the construct would harbour the important regulatory *cis*-elements and would faithfully recapitulate the regulation of the endogenous counterpart. Furthermore, we included a 3'-UTR-less constructs in these experiments as a negative control for the 3'UTR-mediated effects. Indeed, when we transfected these constructs into HepG2 cells, we observed a significant decrease in reporter activity in the presence of miR-124, miR-346, miR-431, miR-506, and miR-708 (Figure 5.7). These data strongly suggest that the decrease in *CPB2* mRNA abundance and TAFI protein levels observed in the presence of miRNA mimics likely arises due to direct targeting of the *CPB2* 3'-UTR.

In our bioinformatic analysis we discovered that the target site for miR-124 is in close proximity to the TTP binding site. In fact, it overlaps with it proximally, such that the first two nucleotides of the miR-124 seed sequence are the last two nucleotides of the TTP binding site. In order to further investigate this, we introduced mutations in this region, changing the core consensus TTP binding site from AATAAATTT to AAGAAAGGG (ΔTTP/3'-UTR construct). We have previously established that this mutation is sufficient to abolish TTP binding [36]. Of note, the activity of the ΔTTP/3'-UTR reporter was significantly higher compared to the *CPB2/*3'-UTR reporter, in which the binding site for TTP remained intact. In the presence of miR-124 mimic, we observed a significant decrease in reporter activity of the wild-type *CPB2*/3'UTR reporter, while the ΔTTP/3'-UTR reporter was refractory to this decrease (Figure 8A). Therefore, the TTP binding site mutation was sufficient to abolish miR-124 binding as well. Additionally, we also determined reporter activity per unit mRNA and found it to be decreased in the presence of miR-124 mimic for the *CPB2*/3'-UTR reporter, but not the ΔTTP/3'-UTR reporter, thus indicating that miR-124 decreased translation efficiency of the *CPB2* transcript (Figure 5.8 B and C). These data also suggest a possible functional

interplay between TTP and miR-124. The interaction and co-operativity between *trans*-acting factors has been described previously, and in case of the mRNA encoding tumor necrosis factor alpha (TNFα), it involves TTP and miR-16 [46]. Interestingly, the interaction between TTP and miR-16 is not a direct one, but rather involves formation of a complex between TTP and Ago/eiF2C family proteins that associate with miR-16. Thus, TTP appears to assist miR-16 in targeting the 3'-UTR of TNFα. In case of *CPB2* 3'UTR and miR-124, this may also be the case. Since the overlap between the binding sites of miR-124 and TTP is quite small (only 2 nucleotides) it seems unlikely that the binding of one would obstruct the binding of another through steric hindrance. It is easy to envision that the binding of TTP and miR-124 may be cooperative, unless the binding induces secondary structure perturbations, as has been described previously [47]. In an event that binding of TTP induces RNA secondary structure changes; this may serve as a mechanism to block miR-124 binding, and *vice versa*, such as occurs in case of HuR and miR-122 in human liver cancer cells [48]. In case of HuR this is expected to be a competitive mechanism, in keeping with the role of HuR as an mRNA stabilizing protein and that of miRNA as an mRNA degrading factor. Due to functional redundancy of TTP and miR-124, it is unlikely that these two factors would act in a competitive manner. TTP binds to the ARE sequences within target mRNAs via its zinc finger [49], and recruits the components of the exosomes, which are also associated with helicase proteins (such as DexH box) that facilitate mRNA deadenylation and decay in mammalian cells [50, 51]. TTP also associates with the eiF2C/Ago family proteins [46], which are a component of the RISC complex. Therefore, it appears that ARE-binding proteins, miRNAs, deadenylase and the exosomes act cooperatively to regulate mRNA degradation. Our data and the evidence from the literature are suggestive of a model in which binding of

TTP is the initial event, followed by a transient interaction with the RISC complexes that scan the 3'-UTR. Such a two-pronged mechanism would ensure the specific targeting of miR-124 to the 3'-UTR of *CPB2*: 1) interaction between TTP and the components of RISC (serving as a docking mechanism), and 2) Watson-Crick base pairing between miR-124 and its recognition sequence within *CPB2* 3'-UTR.

Another interesting observation that arose during our bioinformatic analysis of *CPB2* 3'UTR was that the recognition sequence of miR-143 was predicted to encompass the position +1583, which was previously identified as a location of an A/T polymorphism that is present in the population [35]. Moreover, the T SNP at this position was found to be associated with decreased plasma TAFI concentrations. Previously our group conducted a functional analysis of the effects of 3'-UTR SNPs on expression of *CPB2*, investigating the effects on mRNA stability [22]. The +1583 T SNP was found to result in increased mRNA stability in one haplotype (+1344G /+1542 C/ +1583 T) compared to +1344G /+1542 C/ +1583 A haplotype, whereas it decreased mRNA half-life by 2-fold when combined with + 1542 G SNP (+1344 G/+1542 G/ +1583 T haplotype). Therefore, it is clear that other factors, in addition to the effects mediated by 3'-UTR SNPs, must contribute to modulation of mRNA stability, since the +1583 T SNP does not always alter the stability but is associated with decreased TAFI plasma concentrations. Perhaps some of the additional effects are executed by miR-143, which may alter translation efficiency of the *CPB2* transcript without affecting stability and abundance. MicroRNAs can exert their downregulatory effects either through mRNA degradation or by affecting translation efficiency. The latter likely arises when miRNA-mRNA base pairing contains mismatches, such as the case with miR-143 and its recognition site within *CPB2* 3'-UTR (Figure 5.1). We observed no effect of miR-143

mimic on the endogenous *CPB2* mRNA and TAFI protein levels, as well as on luciferase reporter activity. DNA sequencing revealed that HepG2 cells and the reporter construct contain the A allele at +1583 position. We then performed site directed mutagenesis and introduced T+ 1583 change in the context of the luciferase reporter construct. This resulted in a significant decrease in reporter activity (Figure 5.9), suggesting that indeed the T+ 1583 is required for targeting by miR-143. This may arise due to the position of the SNP within the miR-143 recognition sequence. It is flanked by two mismatches, and if the A+ 1583 mismatch is present as well, the interaction between the miR-143 and its binding site may be very weak and possibly unlikely [27]. Taken together, our data provide preliminary evidence that T +1583 SNP may be the determining factor in making *CPB2* 3'-UTR susceptible to miR-143 targeting, providing novel insights into the mechanistic basis for decreased TAFI plasma concentrations associated with this SNP.

In summary, our data demonstrate that *CPB2* mRNA is susceptible to regulation by the microRNA pathway in HepG2 cells. The ability of Ago2 to specifically associate with *CPB2* 3'-UTR strongly supports this idea. We observed that miR-124, miR-506 and miR-708 decrease TAFI protein levels, owing to a concomitant decrease in *CPB2* transcript abundance. That this effect is mediated by *CPB2* 3'-UTR was also confirmed using luciferase reporter constructs, whose activity was specifically decreased in the presence of miRNA mimics. Moreover, we uncovered a potential interplay between miR-124 and TTP and found that miR-124 decreases translation efficiency whereas TTP likely only affects mRNA stability, without having a prominent effect on translation rate. This study also revealed a plausible mechanistic basis behind plasma TAFI-lowering effect of T+ 1583 SNP, via selective targeting by miR-143. Since the levels of expression of various microRNAs varies across different tissues- and we can certainly attest to this, as

we observed this difference among HepG2 cells, THP-1 monocytes and THP-macrophages - it remains to be explored whether this difference has any functional consequences in terms of tissue-specific *CPB2* gene expression regulation and the amount of TAFI protein produced by different cell types. Moreover, since the levels of expression of many miRNAs can be altered in certain pathologies, ranging from inflammation [52], to various forms of liver [53] and cardiovascular diseases [54], it is plausible that this dysregulation could contribute to changes in TAFI levels, with potential deleterious effects on the fibrinolytic system.

5.5 References

1. Nesheim, M., *Thrombin and fibrinolysis.* Chest, 2003. **124**(3 Suppl): p. 33S-9S.
2. Leung, L.L., et al., *Regulation of tissue inflammation by thrombin-activatable carboxypeptidase B (or TAFI).* Mol Immunol, 2008. **45**(16): p. 4080-3.
3. Bajzar, L., R. Manuel, and M.E. Nesheim, *Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor.* J Biol Chem, 1995. **270**(24): p. 14477-84.
4. Bajzar, L., J. Morser, and M. Nesheim, *TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex.* J Biol Chem, 1996. **271**(28): p. 16603-8.
5. Mao, S.S., et al., *Characterization of plasmin-mediated activation of plasma procarboxypeptidase B. Modulation by glycosaminoglycans.* J Biol Chem, 1999. **274**(49): p. 35046-52.
6. Wang, W., et al., *A study of the mechanism of inhibition of fibrinolysis by activated thrombin-activable fibrinolysis inhibitor.* J Biol Chem, 1998. **273**(42): p. 27176-81.
7. Schneider, M. and M. Nesheim, *A study of the protection of plasmin from antiplasmin inhibition within an intact fibrin clot during the course of clot lysis.* J Biol Chem, 2004. **279**(14): p. 13333-9.
8. Schneider, M., et al., *Activated thrombin-activatable fibrinolysis inhibitor reduces the ability of high molecular weight fibrin degradation products to protect plasmin from antiplasmin.* J Biol Chem, 2004. **279**(14): p. 13340-5.
9. Campbell, W.D., et al., *Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N.* Microbiol Immunol, 2002. **46**(2): p. 131-4.
10. Hendriks, D., et al., *Human serum carboxypeptidase U: a new kininase?* Agents Actions Suppl, 1992. **38 (Pt 1)**: p. 407-13.
11. Du, X.Y., et al., *Regulation of chemerin bioactivity by plasma carboxypeptidase N, carboxypeptidase B (activated thrombin-activable fibrinolysis inhibitor), and platelets.* J Biol Chem, 2009. **284**(2): p. 751-8.
12. Frere, C., et al., *Quantification of thrombin activatable fibrinolysis inhibitor (TAFI) gene polymorphism effects on plasma levels of TAFI measured with assays insensitive to isoform-dependent artefact.* Thromb Haemost, 2005. **94**(2): p. 373-9.
13. van Tilburg, N.H., F.R. Rosendaal, and R.M. Bertina, *Thrombin activatable fibrinolysis inhibitor and the risk for deep vein thrombosis.* Blood, 2000. **95**(9): p. 2855-9.
14. Meltzer, M.E., et al., *Venous thrombosis risk associated with plasma hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1.* Blood, 2010. **116**(1): p. 113-21.
15. Eichinger, S., et al., *Thrombin-activatable fibrinolysis inhibitor and the risk for recurrent venous thromboembolism.* Blood, 2004. **103**(10): p. 3773-6.
16. Leebeek, F.W., et al., *High functional levels of thrombin-activatable fibrinolysis inhibitor are associated with an increased risk of first ischemic stroke.* J Thromb Haemost, 2005. **3**(10): p. 2211-8.

17. Rooth, E., et al., *Thrombin activatable fibrinolysis inhibitor and its relationship to fibrinolysis and inflammation during the acute and convalescent phase of ischemic stroke.* Blood Coagul Fibrinolysis, 2007. **18**(4): p. 365-70.
18. Ladenvall, C., et al., *Thrombin activatable fibrinolysis inhibitor activation peptide shows association with all major subtypes of ischemic stroke and with TAFI gene variation.* Arterioscler Thromb Vasc Biol, 2007. **27**(4): p. 955-62.
19. de Bruijne, E.L., et al., *The role of thrombin activatable fibrinolysis inhibitor in arterial thrombosis at a young age: the ATTAC study.* J Thromb Haemost, 2009. **7**(6): p. 919-27.
20. Tregouet, D.A., et al., *Activated thrombin activatable fibrinolysis inhibitor levels are associated with the risk of cardiovascular death in patients with coronary artery disease: the AtheroGene study.* J Thromb Haemost, 2009. **7**(1): p. 49-57.
21. Frere, C., et al., *Fine mapping of quantitative trait nucleotides underlying thrombin-activatable fibrinolysis inhibitor antigen levels by a transethnic study.* Blood, 2006. **108**(5): p. 1562-8.
22. Boffa, M.B., et al., *Effect of single nucleotide polymorphisms on expression of the gene encoding thrombin-activatable fibrinolysis inhibitor: a functional analysis.* Blood, 2008. **111**(1): p. 183-9.
23. Maret, D., et al., *Role of mRNA transcript stability in modulation of expression of the gene encoding thrombin activable fibrinolysis inhibitor.* J Thromb Haemost, 2004. **2**(11): p. 1969-79.
24. Boffa, M.B., et al., *Acute phase mediators modulate thrombin-activable fibrinolysis inhibitor (TAFI) gene expression in HepG2 cells.* J Biol Chem, 2003. **278**(11): p. 9250-7.
25. Lee, Y., et al., *The nuclear RNase III Drosha initiates microRNA processing.* Nature, 2003. **425**(6956): p. 415-9.
26. Winter, J., et al., *Many roads to maturity: microRNA biogenesis pathways and their regulation.* Nat Cell Biol, 2009. **11**(3): p. 228-34.
27. Bartel, D.P., *MicroRNAs: target recognition and regulatory functions.* Cell, 2009. **136**(2): p. 215-33.
28. Matranga, C., et al., *Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes.* Cell, 2005. **123**(4): p. 607-20.
29. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**(2): p. 281-97.
30. Thomas, M., J. Lieberman, and A. Lal, *Desperately seeking microRNA targets.* Nat Struct Mol Biol, 2010. **17**(10): p. 1169-74.
31. Doench, J.G., C.P. Petersen, and P.A. Sharp, *siRNAs can function as miRNAs.* Genes Dev, 2003. **17**(4): p. 438-42.
32. Adjibade, P. and R. Mazroui, *Control of mRNA turnover: Implication of cytoplasmic RNA granules.* Semin Cell Dev Biol, 2014.
33. Izaurralde, E., *A role for eIF4AII in microRNA-mediated mRNA silencing.* Nat Struct Mol Biol, 2013. **20**(5): p. 543-5.
34. Chu, C.Y. and T.M. Rana, *Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54.* PLoS Biol, 2006. **4**(7): p. e210.
35. Henry, M., et al., *Identification of polymorphisms in the promoter and the 3' region of the TAFI gene: evidence that plasma TAFI antigen levels are strongly genetically controlled.* Blood, 2001. **97**(7): p. 2053-8.

36. Novakovic, D., et al., *Identification of tristetraprolin as a factor that modulates the stability of the TAFI transcript through binding to the 3'-untranslated region.* J Thromb Haemost, 2012. **10**(5): p. 887-94.
37. Lin, J.H., et al., *Identification of human thrombin-activatable fibrinolysis inhibitor in vascular and inflammatory cells.* Thromb Haemost, 2011. **105**(6): p. 999-1009.
38. Slobodin, B. and J.E. Gerst, *RaPID: an aptamer-based mRNA affinity purification technique for the identification of RNA and protein factors present in ribonucleoprotein complexes.* Methods Mol Biol, 2011. **714**: p. 387-406.
39. Hayes, J., P.P. Peruzzi, and S. Lawler, *MicroRNAs in cancer: biomarkers, functions and therapy.* Trends Mol Med, 2014. **20**(8): p. 460-469.
40. Auwerx, J., *The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation.* Experientia, 1991. **47**(1): p. 22-31.
41. Abe, T., et al., *"Differentiation Induction" culture of human leukemic myeloid cells stimulates high production of macrophage differentiation inducing factor.* Cytotechnology, 1991. **5**(Suppl 2): p. 75-93.
42. Yanagisawa, H., et al., *Constitutive expression of exogenous c-myb gene causes maturation block in monocyte-macrophage differentiation.* Biochim Biophys Acta, 1991. **1088**(3): p. 380-4.
43. Cech, T.R. and J.A. Steitz, *The noncoding RNA revolution-trashing old rules to forge new ones.* Cell, 2014. **157**(1): p. 77-94.
44. Mosnier, L.O., et al., *Identification of thrombin activatable fibrinolysis inhibitor (TAFI) in human platelets.* Blood, 2003. **101**(12): p. 4844-6.
45. Schadinger, S.L., et al., *Secretion and antifibrinolytic function of thrombin-activatable fibrinolysis inhibitor from human platelets.* J Thromb Haemost, 2010. **8**(11): p. 2523-9.
46. Jing, Q., et al., *Involvement of microRNA in AU-rich element-mediated mRNA instability.* Cell, 2005. **120**(5): p. 623-34.
47. Kim, H.H., et al., *HuR recruits let-7/RISC to repress c-Myc expression.* Genes Dev, 2009. **23**(15): p. 1743-8.
48. Bhattacharyya, S.N., et al., *Relief of microRNA-mediated translational repression in human cells subjected to stress.* Cell, 2006. **125**(6): p. 1111-24.
49. Michel, S.L., A.L. Guerrerio, and J.M. Berg, *Selective RNA binding by a single CCCH zinc-binding domain from Nup475 (Tristetraprolin).* Biochemistry, 2003. **42**(16): p. 4626-30.
50. Chen, C.Y., et al., *AU binding proteins recruit the exosome to degrade ARE-containing mRNAs.* Cell, 2001. **107**(4): p. 451-64.
51. Tran, H., et al., *Facilitation of mRNA deadenylation and decay by the exosome-bound, DExH protein RHAU.* Mol Cell, 2004. **13**(1): p. 101-11.
52. Kishore, A., et al., *Novel Insights into miRNA in Lung and Heart Inflammatory Diseases.* Mediators Inflamm, 2014. **2014**: p. 259131.
53. Szabo, G. and S. Bala, *MicroRNAs in liver disease.* Nat Rev Gastroenterol Hepatol, 2013. **10**(9): p. 542-52.
54. Bronze-da-Rocha, E., *MicroRNAs Expression Profiles in Cardiovascular Diseases.* Biomed Res Int, 2014. **2014**: p. 985408.

Chapter 6: General Discussion

The collection of studies presented here was aimed at elucidating the mechanisms of gene expression regulation of *CPB2*, the gene encoding human TAFI, with a particular focus on post-transcription control. In Chapter Two, we identified the presence of *cis*-regulatory elements within the 3'-UTR of *CPB2* mRNA, and found that this region harbours one stability element located upstream of the first polyA site, followed by three instability elements, located upstream of the first, second and third polyA sites, respectively. Furthermore, we uncovered a novel mechanism for regulation of *CPB2* mRNA stability by the *trans*-acting protein factor TTP, which recognizes a binding site within the most distal instability element, between the last two polyA sites. TTP is an mRNA destabilizing factor that dictates the stabilities and abundances of many transcripts, including those encoding inflammatory mediators, oncogenes and transcription factors [1]. The role for TAFI in mediating molecular connections between coagulation and inflammation has been suggested previously [2], and we sought to further characterize it through assessment of regulation of *CPB2* expression by inflammatory mediators. It appears that the role of TAFI in these processes is multifaceted, since TAFIa acts on pro-inflammatory factors to inactivate them (C3a, C5a, bradykinin) [3, 4], on anti-inflammatory factors to activate them (plasmin-cleaved chemerin) [5]. Moreover, TAFI expression is itself modulated by the inflammatory mediators, as we described in Chapter Three. We uncovered that pro-inflammatory cytokines and mediators TNFα, IL-6 in combination with IL-1β and LPS decreased TAFI protein production, owing to a reduction in mRNA stability, using our *in vitro* system in HepG2 cells. Furthermore, we delineated the role for TTP in mediating transcript destabilization in the presence of pro-inflammatory mediators. Anti-inflammatory cytokine IL-10 increased both TAFI protein

and *CPB2* mRNA abundance levels, owing to a 2-fold increase in mRNA half-life. These effects however do not appear to be TTP mediated, and may involve other components of the ribonucleoprotein complex that populates the 3'-UTR of *CPB2* mRNA. We also identified HuR as a novel component of this interactome (Figure 6.1). HuR is an RNA-binding protein that acts in antagonistic manner to TTP, and competition between HuR and TTP at the 3'-UTR of TNFα transcript has been identified [6]. This might be a commonly occurring phenomenon, and may operate in the case of *CPB2* mRNA in the context of the inflammatory environment, since we found that the occupancy of the 3'-UTR by HuR was decreased in the presence of inflammatory mediators.

One of the most intriguing findings that originated from our laboratory has been the discovery of *CPB2* expression in THP-monocytes and in THP-1 macrophages derived from differentiation of monocytes [7]. However, little is known about *CPB2* gene expression regulation in cell types other than hepatocytes. In Chapter Four, we initiated the investigation of the regulatory mechanisms that operate in THP-1 macrophages on *CPB2* expression. Interestingly, we found that inflammatory mediators have the opposite effect on *CPB2* mRNA stability, abundance, and TAFI protein levels compared to the one produced in HepG2 cells. These findings may be indicative of tissue-specific *CPB2* expression regulation. Moreover, we observed that polyA site selection is also differently regulated in HepG2 cells and THP-1 macrophages, further supporting this hypothesis.

We found another component of the post-transcriptional regulation machinery to be playing a role in regulation of C*PB2* expression. In Chapter Five, a novel role for microRNA pathway was described in modulating *CPB2* mRNA abundance and TAFI protein levels in HepG2 cells. This study also uncovered preliminary evidence for potential functional interplay between the components of the post-transcriptional

microRNA-ome and proteome, namely for miR-124 and TTP, as well as a potential mechanistic basis for the association between a polymorphism that occurs in the 3'-UTR in the human population, T+ 1583A, with lower plasma TAFI concentrations in individuals bearing the T allele [8] (Figure 6.1).

Taken together, our analyses of regulation of *CPB2* expression at the post-transcriptional level in liver cells and THP-1 macrophages have provided important fundamental, mechanistic insights into how expression of this important haemostatic factor is modulated. This new knowledge is relevant not only to rationalizing the clinical studies on variation of plasma TAFI concentrations in the human population, but also to understanding how gene regulatory events may be an essential component of the TAFI system. These analyses provide a solid platform for studies of the role of TAFI pathway in health and disease, and in cell types that may be involved in deranged haemostasis and inflammation.

6.1 Post-transcriptional control: crucial regulator of *CPB2* gene expression

Translation of mRNA into proteins can be specifically regulated by a combinatorial action of RNA-binding factors (proteins and antisense RNAs), that can affect mRNA stability, localization, translation initiation and elongation. Untranslated regions at the 3' end (3'-UTRs) of mRNAs are involved in precise orchestration of these events by virtue of specific sequences, called *cis*-elements that serve as sites of interaction with the various *trans*-acting factors. The importance of 3'-UTRs was largely underestimated until the discovery of ARE-binding proteins, and even more so, until the discovery of microRNAs. Moreover, it is becoming increasingly apparent that the 3'-UTR binding factors do not act separately, but rather in cooperation, and this crosstalk is mediated by binding sequences or direct protein-protein, or protein-miRNA interactions.

Accumulating evidence suggests that many RNA binding factors share common target 3'-UTRs, and can bind to non-overlapping sites, or to common sequences in a competitive fashion [6]. The outcome of the cooperative function of the factors bound to the common 3'-UTR depends on several circumstances, such as the levels of their expression, their localization, and their affinity towards and availability of their binding site. The situation becomes further complicated by the fact that these circumstances can be modulated by physiological and pathological processes.

One of the best characterized *cis*-elements is the cytoplasmic polyadenylation element (CPE) located in close proximity to the canonical polyA hexanuclotide AAUAAA [9]. CPE-binding protein (CPEB) is an ARE-binding protein that binds CPE and regulates specific target mRNAs, directly controlling physiological processes in mammalian cells, such as cell cycle progression, particularly during senescence, suggesting a role in cancer and aging. Other well characterized ARE-binding protein factors include Hu/ELAV family of proteins, HuR (which is ubiquitously expressed), and HuB, -C and –D, which have evolved to acquire specialized functions, and are primarily neuronal. HuR mainly acts on target 3'-UTRs and enhances transcript stability or translation rate, although in some instances acts as an mRNA destabilizer [10-12]. AU-binding factor 1 (AUF1), belongs to a big family of hnRNPs that includes hnRNP A, B, C, D, E, F, H, I, K, L, M, Q and R. AUF1 primarily targets mRNAs for degradation, most likely by recruiting them to exosomes for degradation [13, 14]. However, it was found to cause the opposite effect – enhancing stability and translation – in the case of some mRNAs [15]. It is evident from these examples that cooperation and crosstalk among the components of the 3'-UTR interactome decide the final fate of the transcript.

In the case of *CPB2* 3'-UTR, we identified TTP, as the *trans*-acting protein factor that dictates transcript stability, and is involved in mediating regulated changes in *CPB2* mRNA and TAFI protein abundance in the presence of inflammatory mediators. TTP exerts these effects through control of *CPB2* mRNA stability. TTP has been characterized as an important regulator of initiation and resolution of inflammatory responses, through its rigorous control of mRNA stability of transcripts encoding many cytokines, including COX-2 [16, 17] and the master cytokine TNFα [18]. The fact that *CPB2* mRNA belongs to the pool of transcripts regulated by TTP underscores the involvement of TAFI in regulation of inflammation. In fact, TAFI appears to play a multidimensional role in regulation of inflammation: TAFI downregulates inflammation by virtue of inactivation of pro-inflammatory peptides and TAFI expression is itself downregulated by the inflammatory cytokines, as we described Chapter Three. Additionally, we found that the functional antagonist of TTP, HuR, may also assist in this process, and may serve as a supplementary mechanism to downregulate *CPB2* transcript and TAFI protein levels in an inflammatory environment. Therefore, this TTP-HuR regulatory axis serves as an example of cooperation among RNA-binding factors that ultimately determines the fate of *CPB2* mRNA.

Although we initially speculated that the binding of TTP and HuR may be competitive, this does not appear to be the case. In keeping with the role of HuR as an mRNA stabilizing factor, we speculate that it likely contacts the stability *cis*-element that is located upstream of the last instability element that TTP binds, a hypothesis that would be in line with the observed data.

6.2 *CPB2* gene expression regulation in THP-1 macrophages: evidence for existence of tissue-specific regulatory mechanisms

Another particularly interesting characteristic of the *CPB2* 3'-UTR is that is possesses three possible polyA signals. All of these are in fact utilized, as we previously determined for HepG2 cells, albeit with different frequencies [19]. It appears that alternative polyadenylation is a widespread phenomenon, and can affect tissue specific expression, mRNA localization, mRNA decay and translation efficiency [20-24], and may play a role in disease states [25]. Here we uncovered a different polyadenylation pattern of *CPB2* mRNA in THP-1 macrophages, compared to that in HepG2 cells. The regulatory function of alternative polyadenylation lies in formation of RNA-binding factor-deficient or -proficient 3'-UTR, serving as a module to promote or prevent association of the particular RNA-binding factor and its target site. Approximately half of the pool of *CPB2* transcripts in THP-1 macrophages consists of the longest polyA form, that harbours the TTP binding site, which is in contrast to HepG2 cells that under basal conditions rarely utilize the last polyA site and the longest polyA form only accounts for 2% of the total *CPB2* transcripts [19]. In keeping with our previous finding that the longest transcript is the least stable, we speculate that such high concentration of this longest form contributes to the shorter half-life of *CPB2* mRNA endogenously expressed by THP-1 macrophages (2.1 hour, compared to 3.1 hour in HepG2 cells). A notable example from the literature is COX-2 mRNA, that contains two alternative polyA sites, and the use of the more distal site is more frequent and results in inclusion of the TTP binding site [16]. This pattern of alternative polyadenylation is deranged in cancer, such that the first polyA site is favoured, resulting in exclusion of the TTP binding site and increased COX-2 expression [17].

In Chapter Four, we investigated the influence of inflammatory mediators on the frequency of usage of the three polyA sites in THP-1 macrophages. We have previously shown that when IL-6 and IL-1β are administered in combination to HepG2 cells this results in preferential formation of the longest transcript that is further destabilized by 50% [19]. With the discovery of the functional TTP binding site between the last two polyA sites, it is tempting to speculate that increased frequency of the usage of the last polyA site in the presence of this stimulus is the operating mechanism behind this destabilization. In THP-1 macrophages however, we found that the usage of this last polyA site decreases in the presence of the inflammatory mediators, which may at least in part account for increased mRNA stability of the endogenous *CPB2* transcript. Also, this effect appears to be much less dramatic in THP-1 macrophages compared to HepG2 cells. Preferential formation of the shortest transcript in the presence of inflammatory mediators would result in the enrichment of TTP-binding site-deficient transcripts which have longer half-lives. In addition to TTP, HuR may also play a role in exerting this effect. As we demonstrated for HepG2 cells in Chapter Three, the absence of TTP binding site increases the HuR occupancy of fusion transcripts containing the TAFI 3'-UTR in the presence of inflammatory mediators. In keeping with the stabilizing effect of the first *cis*-element we described in Chapter Two, it is tempting to speculate that this region also constitutes the HuR binding site. Therefore the increased production of HuR-proficient, TTP-deficient transcripts by THP-1 macrophages in the presence of inflammatory mediators is expected to result in increased overall abundance of the TAFI mRNAs and subsequent increase in TAFI protein levels, as we described in Chapter Four.

In case of inflammation, macrophages can assume either an inflammatory or an anti-inflammatory role. Inflammatory macrophages, referred to as M1 macrophages, are

known to react strongly to invading pathogen components (such as LPS) or other danger signals (IFN-γ and TNFα) by producing pro-inflammatory cytokines, inducible nitric oxide synthases and reactive oxygen species (ROS) [26]. M1 macrophages are predominantly active in the initial stages of acute inflammation (such as upon bacterial infection) or in the later phase of chronic inflammation (as occurs in the vessel wall in atherosclerosis) [27]. While they exert most of their functions locally, the secreted pro-inflammatory cytokines act as chemoattractants for T cells and natural killer cells, recruited to aid in the inflammatory process [28]. In situations when M1 macrophages are unable to cease their production of inflammatory mediators, uncontrolled inflammation ensues, followed by tissue damage as a result of excessive ROS production and recruitment of other immune cells, a hallmark of many autoimmune diseases.

Similarly, in atherosclerosis, activated M1 macrophages engulf the surrounding lipid molecules, becoming foam cells that contribute substantially to atherosclerotic plaque formation through potentiation of pro-inflammatory environment in the immediate vicinity of the plaque. The regulation of *CPB2* gene expression in this milieu argues in favour for the role of TAFI as pro-atherogenic factor. In this context, enhanced expression of TAFI through the usage of the more proximal polyA site is expected to increase the TAFI load within and around the atherosclerotic plaque, resulting in attenuation of pericellular plasminogen activation, increased extracellular matrix deposition and increased smooth muscle cell migration. Therefore, the role of macrophage-secreted TAFI in the context of the vessel wall might be centered primarily on regulation of the plasminogen system. Since the primary role of liver-produced TAFI is in fibrinolysis, it is not surprising that gene expression regulation at these two locales is different, and these changes appear to occur at the level of post-transcriptional regulation.

Regulation at this level is possibly most efficient and energetically favourable for the cell, since the factors involved are present in the same compartment as the transcript (or are easily transported), compared to changes at the transcriptional level, which would involve much more complex organization of the transcription machinery and the events thereafter. Taken together, our studies of post-transcriptional regulation of *CPB2* expression in THP-1 macrophages, both constitutive and regulated, will provide important context for further studies of tissue-specific gene expression regulation of *CPB2*.

6.3 'Micro-management' of *CPB2* gene expression regulation: evidence for the role of the microRNA pathway

MicroRNAs are a class of small non-coding regulatory RNA molecules that represent one of the major breakthroughs in cellular and molecular biology. MiRNAs originate from RNA polymerase II-transcribed precursors, which are processed by Drosha enzyme in the nucleus, generating pre-miRNA precursors [29]. Further processing occurs in the cytoplasm after export of pre-miRNAs, and is executed mainly by Dicer, that is responsible for formation of ~22nt miRNA duplexes [30]. The functional unit of a miRNA is the guide strand, that associates with the RISC complex, and that possesses complimentary sequence to its target sequence in the 3'-UTR of protein-coding mRNAs, whereas the other strand (passenger strand) is degraded . At only about 22 nt in length, these tiny molecules play key roles in almost all cellular processes [30-32]. Since a single miRNA molecule can target many different mRNAs, alterations of miRNA levels expressed by the cell may essentially influence cellular homeostasis, and in most extreme cases result in pathological consequences, such as malignant transformation or cell death [33, 34]. In fact, the evidence for the involvement of miRNAs in tumorogenesis emerged

over a decade ago [33]. Since then, multiple pathophysiological effects have been observed when miRNA expression levels are deregulated [35-37].

In support of our findings of tissue-specific *CPB2* gene expression regulation, we also observed that the expression levels of several candidate miRNAs differ between THP-1 macrophages and HepG2 cells. In Chapter Five, we described the potential of these candidate miRNAs, miR-124, miR-346, miR-431, miR-506 and miR-708, to downregulate *CBP2* mRNA and TAFI protein levels in HepG2 cells. Furthermore, we confirmed that the 3'-UTR of *CPB2* transcript is a functional target for these miRNAs with our luciferase reporter system. These studies will be particularly informative for future studies of miRNA involvement in other cell types where *CPB2* is expressed, such as in THP-1 monocytes, THP-1 macrophages, megakaryocytes and platelets.

Further evidence of cooperativity between RNA regulatory factors, namely RNA-binding proteins and miRNAs, also arose from the data we presented in Chapter Five. The target site for miR-124 was of particular interest to us, as we discovered that it lies in close vicinity to the TTP binding site within *CBP2* 3'-UTR. Upon closer inspection, we established that the two sites overlap by two nucleotides, where the TTP binding site is located proximally to the miR-124 target site. We employed our luciferase reporter system to further confirm this, and found that when the TTP binding site is mutated, miR-124 targeting of the *CPB2* 3'-UTR is also abolished. Furthermore, we found that miR-124 decreases translation efficiency, whereas TTP does not seem to play a role in this process. Therefore, the combinatorial action of these two RNA binding factors may be at play in determination of *CPB2* mRNA fate. It is remains to be elucidated whether increased targeting of TTP, such as occurs in the presence of inflammatory mediators, also results in increased miR-124 targeting of the *CPB2* 3'-UTR. It is also presently unknown

whether the same regulatory paradigm extends to other cell types where *CPB2* is expressed.

Multiple studies have shown that plasma concentrations of TAFI vary significantly in the population [38]. There are a number of SNPs throughout the *CPB2* and altogether account for 25 % of the variation in plasma TAFI levels [39]. Of the SNPs that occur in the 3'-UTR, of particular interest to us was T+ 1583A SNP, mainly because we discovered that it is encompassed within the target site of miR-143. Furthermore, miR-143 complementary base pairing was predicted to occur when the T allele was present at this position. The T+ 1583A SNP has been associated with lower TAFI plasma concentrations [39], and when present in certain haplotype combinations, but not on its own, it resulted in destabilization of *CPB2* mRNA [40]. We hypothesized that the selective targeting by miR-143 may at least in part account for the mechanism behind these effects. After genotyping HepG2 cells and the 3'-flanking region of our luciferase reporter system, we discovered that they contain the A allele at +1583 position, which may explain why our initial studies demonstrated miR-143 ineffective in downregulation of *CPB2* mRNA and TAFI protein levels. We selectively introduced the T SNP at this position with site directed mutagenesis in the context of the luciferase reporter constructs, and observed decrease in reporter activity in the presence of miR-143 mimic, while the A allele-bearing construct was refractory to this downregulation. This study was particularly informative in delineating new mechanisms for *CPB2* gene expression regulation by both genetic and gene-regulatory factors. More fundamentally, it also provides evidence for the involvement of the miRNA in regulation of gene expression by single nucleotide polymorphisms, and this may be widely occurring phenomenon in regulation of other genes.

6.4 Implications for further research

The activity of TAFIa in the plasma compartment reduces fibrin clot lysis and contributes to clot stability. Therapeutic thrombolysis consists of pharmacological dissolution of the clot by injection of plasminogen activators (such as tPA) that activate the fibrinolytic system. This intervention is commonly used for patients with myocardial infarction and ischemic stroke with great clinical benefit. However, the thrombolytic agents currently used all have significant shortcomings, ranging from the need for large doses and limited specificity, to associated bleeding complications and reocclusions [41]. Therefore, adjunctive therapy that would potentiate the tPA-mediated fibrinolysis would have a tremendous benefit in erasing some of the shortcomings, such as reducing the dose of plasminogen activators and the associated bleeding tendencies. Indeed, the potential for TAFIa inhibition as adjuvant therapy has been investigated in a rabbit model of arterial thrombolysis in which TAFIa-specific inhibitor, PTCI, was used. The study demonstrated enhancement of several parameters associated with improved thrombolysis when PTCI and tPA were co-administered [42].

Additional avenues for modulation of TAFIa activity can also occur at the level of gene expression regulation. Antisense oligonucleotides, small interfering RNAs (siRNAs), and microRNAs (miRNAs) are emerging as important therapeutic modalities for the treatment of cardiovascular diseases (reviewed in [43]). Two strategies have evolved to alter miRNA activity, the first using synthetic mimicry of naturally occurring miRNAs, while the second blocks activity via complementary antisense oligonucleotide inhibitors [44]. In Chapter Five, we described that *CPB2* transcript is a target of several miRNAs. Therefore, the alteration of the activities of the specific miRNAs is a plausible therapeutic strategy to modulate TAFI protein levels.

Evaluation of the *in vivo* efficacy of such microRNA-based therapy would require the use of an appropriate animal model. Mice are the mainstay of *in vivo* experimentation and in many respects their biology is remarkably similar to that of the human. Sequencing of the human and mice genomes revealed that only approximately 300 genes appear unique to one species or the other [45]. Despite such high degree of conservation, significant differences exist for many biological systems. One such example is the immune system, which in mice and humans differs in many respects, including development, activation, and response to challenge [46]. This should not come as a surprise, provided that not only mice and humans differ significantly in size and lifespan, these two species have also been presented with widely different pathogenic challenges during evolution in their respective ecological niches.

Regulation of *CPB2* expression in mice and humans also appears to be different. A recent report from our laboratory revealed that gene expression regulation of *CPB2* differs substantially in the presence of inflammatory mediators [47]. We observed an increase in *CPB2* mRNA abundance upon treatment of primary mouse hepatocytes or the mouse hepatic cell line FL83B with TNFα, and we revealed that the mechanistic basis for this increase lies in NFκB-mediated upregulation of promoter activity. In Chapter Three we described the opposite effect on both mRNA and TAFI protein abundance in human hepatic cell line, mediated by TTP-induced destabilization of the transcript and subsequent decrease in TAFI protein levels. Therefore, the same stimulus appears to regulate *CPB2* expression in different ways and via different mechanisms in the two species. More fundamentally, this finding also reflects the fact that key regulatory sequences involved in transcription are not conserved between the mouse and the human, as the human promoter lacks the consensus NFκB binding site responsible for cytokine

induction of the mouse *CPB2* promoter. In further support of this notion, unpublished data from our laboratory revealed that the 3'-UTR of the mouse *CPB2* transcript shares homology woth the human counterpart only in the first 100 nucleotides. The mouse 3'-UTR contains only two alternative polyA sites, compared to the three in the human counterpart, and these occur within 120 nucleotides of the stop codon. It is therefore not unexpected that there is differential regulation at both the transcriptional and post-transcriptional levels, particularly pertaining to the inflammatory milieu. It appears that evolutionarily challenges presented to humans resulted in the acquirement of additional *cis*-regulatory elements within *CPB2* 3'-UTR, and the loss of transcription factor binding sites within the *CPB2* promoter. Perhaps this evolved as a more sophisticated strategy to fine-tune *CPB2* gene expression in response to inflammatory stimuli. Alternatively, the divergence in the regulatory regions of *CPB2* may attest to the differences between primates and rodents with respect to the role of TAFI in host defense. Therefore, a mouse model may not be most suitable to use in studies elucidating the function of the TAFI pathway in inflammation.

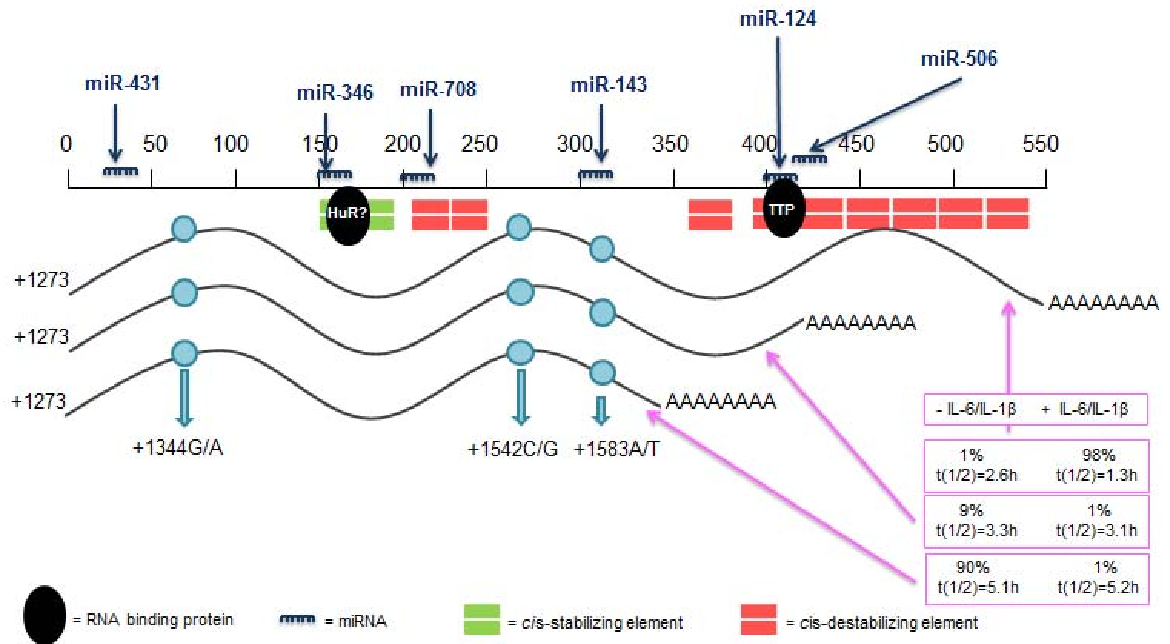

Figure 6.1. Putative components of the miRNA-ribonucleoprotein complex involved in metabolism of *CPB2* mRNA. 3'-UTR of *CPB2* mRNA with three potential polyA sites and the respective transcripts. Location of SNPs is shown by blue spheres. Intrinsic stabilities and abundances of the three polyA forms in HepG2 cells under basal conditions and in the presence of IL-6 and IL-1β are indicated in purple boxes. Question mark emphasizes the unknown nature of the particular interaction.

6.5 Concluding remarks

The impact of the mechanistic studies of gene expression regulation is at least three-fold. Firstly, these studies delineate key regulatory mechanisms that are employed for both constitutive and regulated expression of the gene under study. Secondly, they pinpoint the key factors and players that act together to orchestrate this regulation. This is particularly important for complex regulatory environments, such as the case with inflammation. Lastly, in the pathological milieu, they illuminate the possible mechanisms/factors that may be involved and a detailed understanding of these mechanisms may aid in development of new tools for diagnostics and treatment strategies. Ultimately, elucidation of these mechanisms and events may lead to discovery of new roles for the gene product in question, and more importantly reveal key evolutionarily insights that may apply to other genes.

With the studies described here, we have gained new insights into the regulation of *CPB2* expression in HepG2 cells and THP-1 macrophages that particularly underscore the key role for post-transcriptional events. Of the specific RNA-regulatory factors, we described the roles for TTP, HuR, miR-124, miR-143, miR-346, miR-506 and miR-708 in hepatic cells (Figure 6.1). Whether the same regulatory network operates in other cell types where *CPB2* is expressed (i.e. monocytes, macrophages, megakaryocytes and platelets) still remains to be elucidated. The work described in this dissertation thus provides a solid platform for the design of similar studies aimed at elucidating gene regulatory events of *CPB2* at these sites. Such studies are essential in understanding the full capacity of the TAFI pathway functions in health and disease. More fundamentally, new knowledge gained from these studies would provide key insights into the gene

regulatory mechanisms that operate at tissue-specific level to fine-tune the levels of the protein product and/or uncover its novel roles.

6.6 References

1. Blackshear, P.J., *Tristetraprolin and other CCCH tandem zinc-finger proteins in the regulation of mRNA turnover.* Biochem Soc Trans, 2002. **30**(Pt 6): p. 945-52.
2. Colucci, M. and N. Semeraro, *Thrombin activatable fibrinolysis inhibitor: at the nexus of fibrinolysis and inflammation.* Thromb Res, 2012. **129**(3): p. 314-9.
3. Campbell, W.D., et al., *Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N.* Microbiol Immunol, 2002. **46**(2): p. 131-4.
4. Hendriks, D., et al., *Human serum carboxypeptidase U: a new kininase?* Agents Actions Suppl, 1992. **38 (Pt 1)**: p. 407-13.
5. Du, X.Y., et al., *Regulation of chemerin bioactivity by plasma carboxypeptidase N, carboxypeptidase B (activated thrombin-activable fibrinolysis inhibitor), and platelets.* J Biol Chem, 2009. **284**(2): p. 751-8.
6. Tiedje, C., et al., *The p38/MK2-driven exchange between tristetraprolin and HuR regulates AU-rich element-dependent translation.* PLoS Genet, 2012. **8**(9): p. e1002977.
7. Lin, J.H., et al., *Identification of human thrombin-activatable fibrinolysis inhibitor in vascular and inflammatory cells.* Thromb Haemost, 2011. **105**(6): p. 999-1009.
8. Henry, M., et al., *Identification of polymorphisms in the promoter and the 3' region of the TAFI gene: evidence that plasma TAFI antigen levels are strongly genetically controlled.* Blood, 2001. **97**(7): p. 2053-8.
9. D'Ambrogio, A., K. Nagaoka, and J.D. Richter, *Translational control of cell growth and malignancy by the CPEBs.* Nat Rev Cancer, 2013. **13**(4): p. 283-90.
10. Srikantan, S., K. Tominaga, and M. Gorospe, *Functional interplay between RNA-binding protein HuR and microRNAs.* Curr Protein Pept Sci, 2012. **13**(4): p. 372-9.
11. Srikantan, S. and M. Gorospe, *HuR function in disease.* Front Biosci (Landmark Ed), 2012. **17**: p. 189-205.
12. Chang, N., et al., *HuR uses AUF1 as a cofactor to promote p16INK4 mRNA decay.* Mol Cell Biol, 2010. **30**(15): p. 3875-86.
13. Gratacos, F.M. and G. Brewer, *The role of AUF1 in regulated mRNA decay.* Wiley Interdiscip Rev RNA, 2010. **1**(3): p. 457-73.
14. Chen, C.Y., et al., *AU binding proteins recruit the exosome to degrade ARE-containing mRNAs.* Cell, 2001. **107**(4): p. 451-64.
15. Raineri, I., et al., *Roles of AUF1 isoforms, HuR and BRF1 in ARE-dependent mRNA turnover studied by RNA interference.* Nucleic Acids Res, 2004. **32**(4): p. 1279-88.
16. Sawaoka, H., et al., *Tristetraprolin binds to the 3'-untranslated region of cyclooxygenase-2 mRNA. A polyadenylation variant in a cancer cell line lacks the binding site.* J Biol Chem, 2003. **278**(16): p. 13928-35.
17. Boutaud, O., et al., *Tristetraprolin binds to the COX-2 mRNA 3' untranslated region in cancer cells.* Adv Exp Med Biol, 2003. **525**: p. 157-60.
18. Lai, W.S., et al., *Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA.* Mol Cell Biol, 1999. **19**(6): p. 4311-23.

19. Maret, D., et al., *Role of mRNA transcript stability in modulation of expression of the gene encoding thrombin activable fibrinolysis inhibitor.* J Thromb Haemost, 2004. **2**(11): p. 1969-79.
20. Hesketh, J., *3'-Untranslated regions are important in mRNA localization and translation: lessons from selenium and metallothionein.* Biochem Soc Trans, 2004. **32**(Pt 6): p. 990-3.
21. Edwalds-Gilbert, G., K.L. Veraldi, and C. Milcarek, *Alternative poly(A) site selection in complex transcription units: means to an end?* Nucleic Acids Res, 1997. **25**(13): p. 2547-61.
22. Zhang, H., J.Y. Lee, and B. Tian, *Biased alternative polyadenylation in human tissues.* Genome Biol, 2005. **6**(12): p. R100.
23. Ara, T., et al., *Conservation of alternative polyadenylation patterns in mammalian genes.* BMC Genomics, 2006. **7**: p. 189.
24. Sandberg, R., et al., *Proliferating cells express mRNAs with shortened 3' untranslated regions and fewer microRNA target sites.* Science, 2008. **320**(5883): p. 1643-7.
25. Khabar, K.S., *The AU-rich transcriptome: more than interferons and cytokines, and its role in disease.* J Interferon Cytokine Res, 2005. **25**(1): p. 1-10.
26. Zhang, X. and D.M. Mosser, *Macrophage activation by endogenous danger signals.* J Pathol, 2008. **214**(2): p. 161-78.
27. Mahdavian Delavary, B., et al., *Macrophages in skin injury and repair.* Immunobiology, 2011. **216**(7): p. 753-62.
28. Biswas, S.K. and A. Mantovani, *Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm.* Nat Immunol, 2010. **11**(10): p. 889-96.
29. Lee, Y., et al., *The nuclear RNase III Drosha initiates microRNA processing.* Nature, 2003. **425**(6956): p. 415-9.
30. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**(2): p. 281-97.
31. Bushati, N. and S.M. Cohen, *microRNA functions.* Annu Rev Cell Dev Biol, 2007. **23**: p. 175-205.
32. Bartel, D.P., *MicroRNAs: target recognition and regulatory functions.* Cell, 2009. **136**(2): p. 215-33.
33. Calin, G.A., et al., *Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia.* Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15524-9.
34. Oom, A.L., B.A. Humphries, and C. Yang, *MicroRNAs: Novel Players in Cancer Diagnosis and Therapies.* Biomed Res Int, 2014. **2014**: p. 959461.
35. Bronze-da-Rocha, E., *MicroRNAs Expression Profiles in Cardiovascular Diseases.* Biomed Res Int, 2014. **2014**: p. 985408.
36. Kishore, A., et al., *Novel Insights into miRNA in Lung and Heart Inflammatory Diseases.* Mediators Inflamm, 2014. **2014**: p. 259131.
37. Szabo, G. and S. Bala, *MicroRNAs in liver disease.* Nat Rev Gastroenterol Hepatol, 2013. **10**(9): p. 542-52.
38. Frere, C., et al., *Quantification of thrombin activatable fibrinolysis inhibitor (TAFI) gene polymorphism effects on plasma levels of TAFI measured with assays*

insensitive to isoform-dependent artefact. Thromb Haemost, 2005. **94**(2): p. 373-9.

39. Frere, C., et al., *Fine mapping of quantitative trait nucleotides underlying thrombin-activatable fibrinolysis inhibitor antigen levels by a transethnic study.* Blood, 2006. **108**(5): p. 1562-8.
40. Boffa, M.B., et al., *Effect of single nucleotide polymorphisms on expression of the gene encoding thrombin-activatable fibrinolysis inhibitor: a functional analysis.* Blood, 2008. **111**(1): p. 183-9.
41. Willemse, J.L., et al., *Carboxypeptidase U (TAFIa): a new drug target for fibrinolytic therapy?* J Thromb Haemost, 2009. **7**(12): p. 1962-71.
42. Klement, P., P. Liao, and L. Bajzar, *A novel approach to arterial thrombolysis.* Blood, 1999. **94**(8): p. 2735-43.
43. Lee, R.G., et al., *Antisense technology: an emerging platform for cardiovascular disease therapeutics.* J Cardiovasc Transl Res, 2013. **6**(6): p. 969-80.
44. Esau, C.C. and B.P. Monia, *Therapeutic potential for microRNAs.* Adv Drug Deliv Rev, 2007. **59**(2-3): p. 101-14.
45. Waterston, R.H., E.S. Lander, and J.E. Sulston, *On the sequencing of the human genome.* Proc Natl Acad Sci U S A, 2002. **99**(6): p. 3712-6.
46. Mestas, J. and C.C. Hughes, *Of mice and not men: differences between mouse and human immunology.* J Immunol, 2004. **172**(5): p. 2731-8.
47. Garand, M., et al., *Regulation of the mouse gene encoding TAFI by TNFalpha: role of NFkappaB binding site.* Cytokine, 2012. **57**(3): p. 389-97.

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