FUNCTIONAL STUDIES OF HUMAN CELLULAR DETOXIFICATION ENZYMES

by

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Cellular detoxification allows for the maintenance of cellular homeostasis and prevention of abnormal cell growth by clearing harmful xenobiotics and endobiotics. After oxygenation by phase I enzymes, phase II enzymes such as glucuronosyltransferases and glutathione-s-transferases conjugate a small molecule to the compound, marking it for subsequent export. Many up-stream enzymes are also essential to cellular detoxification by supplying the small compounds for conjugation. These up-stream enzymes include UDP-glucose dehydrogenase, which synthesizes UDP-glucuronate, and glutamate cysteine ligase, which catalyzes the first and rate-limiting step in the synthesis of glutathione.

UDP-glucose dehydrogenase (UGDH) is an important enzyme in human development and in the progression of many types of human epithelial cancers. Recently, mutations in UGDH were identified that are associated with congenital heart defects and cause a shift from a hexameric to a dimeric state. These clinical mutants, along with two engineered dimer mutants were used to examine differences in UGDH function resulting from loss of hexameric structure. The dimer mutants exhibited near wild-type activity *in vitro*, and significant differences in UDP-glucuronate levels were not observed in HEK 293 cells. Despite this, the

phenotype of development defects associated with the UGDH clinical mutants is at least partially explained by a reduction in protein stability.

Glutamate cysteine ligase (GCL) deficiency is a rare autosomal recessive trait that compromises production of glutathione, a critical redox buffer and enzymatic cofactor. Glutamate cysteine ligase is a heterodimer comprised of a catalytic (GCLC) and a regulatory subunit (GCLM). Four clinical missense mutations have been identified within GCLC: Arg127Cys, Pro158Leu, His370Leu, and Pro414Leu. Embryonic fibroblasts from GCLC null mice were transiently transfected with wild-type or mutant GCLC and cellular glutathione levels were determined to be significantly lower in the mutants relative to wild-type. In an *S. cerevisiae* model system, mutant GCLC alone could not complement a glutathione-deficient strain and required the concurrent addition of GCLM to restore growth. Kinetic characterizations of the recombinant GCLC mutants indicated that the Arg127Cys, His370Leu, and Pro414Leu mutants have compromised enzymatic activity that can largely be rescued by the addition of GCLM, while the Pro158Leu mutant has kinetic constants comparable to wild-type GCLC.

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Chapter 1

Introduction

1.1 Cellular detoxification pathways

Cellular detoxification pathways are essential to the maintenance of cellular homeostasis and prevention of disease or progression of abnormal cell growth. Small hydrophobic molecules diffuse freely across the plasma membrane, allowing the entry of potentially harmful levels of xenobiotics or endogenous compounds into the cell. Fortunately, cellular detoxification is both efficient and broadly specific, allowing many different classes of compounds to be effectively cleared from the cell in a 3-step process [1, 2]. First, phase I enzymes (primarily members of the cytochrome 450 family of enzymes) oxygenate a compound, making it an appropriate substrate for phase II enzymes. These enzymes, which include glucuronosyltransferases and glutathione-s-transferases, conjugate a small molecule, such as glucuronate or glutathione, to the compound. These conjugated molecules mark the compound for subsequent export through multi-drug resistance transporters [1, 2].

Besides phase I and phase II enzymes, a multitude of up-stream enzymes participate in cellular detoxification by regulating the availability of small molecules for conjugation to xenobiotics. Thus, UDP-glucose dehydrogenase converts the common metabolite UDP-glucose into UDP-glucuronate, which may then be used as a substrate by UDP-glucuronosyltransferases (UGTs) or in glycosylation reactions in the lumen of the ER [3]. Besides participating in clearance of xenobiotics, UGTs also glucuronidate endogenous compounds such as bilirubin and steroid hormones allowing them to be exported from the cell [4].

Similarly, glutathione has a variety of important cellular fates after sequential synthesis by glutamate cysteine ligase and glutathione synthetase. Glutathione's role as a redox regulator is well recognized [5, 6]. However, glutathione is also used extensively by glutathione-S-transferases to tag compounds for export and metabolize the by-products of oxidative stress [7]. The role of glutathione in cellular detoxification, like that of UDP-glucuronate, is especially critical in the liver, where the rate of detoxification is particularly high and a disproportionate amount of phase I, phase II, and upstream enzymes are synthesized [4].

Because of the critical roles of these two enzymes in supplying metabolites necessary for the detoxification of endobiotics and xenobiotics and their other cellular roles in metabolism and redox homeostasis, UDP-glucose dehydrogenase and glutamate cysteine ligase are ideal candidates for structural studies to reveal the basis of *in vivo* and *in vitro* enzyme function.

1.2 Roles of UDP-glucuronate

UDP-glucuronate is an important cellular metabolite utilized as a precursor in glycosaminoglycan synthesis, protein glycosylation, and cellular detoxification [8,10, 13]. UDP-glucuronate is synthesized from UDP-glucose by the enzyme UDP-glucose dehydrogenase via two successive oxidations to convert the 6' hydroxyl to a carboxylate. UDP-glucuronate has a variety of cellular fates, many of which involve further conversion at the ER or Golgi lumen [9].

UDP-glucuronate is incorporated into glycosaminoglycans in a variety of ways. The enzyme UDP-xylose synthase or UDP-glucuronate decarboxylase, located

in lumen of the ER and Golgi, decarboxylates UDP-glucuronate to form UDP-xylose [10]. UDP-xylose is a precursor for the synthesis of these glycosaminoglycans: heparin, heparin sulfate, chondroitin sulfate and dermatan sulfate [9, 11]. UDP-glucuronate is also incorporated into hyaluronan, another glycosaminoglycan, that is synthesized at the cell surface and extruded into the extracellular space [8]. Glycosaminoglycans may function independently as cellular coating or connective tissue or may be attached to proteins in the late ER or Golgi [9]. Glycosaminoglycan synthesis has been implicated in developmental defects, cancer progression, arthritis, [12]and other disease states.

UDP-glucuronate contributes to protein glycosylation by providing glucuronosyl units and xylose units for sugar transferases. Several types of proteoglycans may be formed. Certain signaling proteins on the extracellular surface are O-glucosylated and xylosated [13]. An example of this is the xylosylation on O-glucose in EGF repeats on the Notch protein [10]. Additionally, synthesis of proteoglycans containing glycosaminoglycans in the late ER and Golgi are initiated by transfer of a core tetrasacharride containing xylose, two galactose units and a glucuronate to a serine in the protein [14, 15]. After transfer of this priming linker, glycosaminoglycan biosynthesis proceeds by addition of constituent sugars by sugar transferases.

Finally, UDP-glucuronate is the substrate for UDP-glucuronosyltransferases, arguably the most important class of the phase II drug metabolizing enzymes. Besides their critical role in detoxifying xenobiotics, UDP-glucuronosyltransferases

also allow cells to remove toxic endobiotics [3, 4]. These enzymes attach the glucuronate moiety to the compound, allowing the conjugate to be exported by multi-drug resistance proteins in the plasma membrane. Thus, UDP-glucuronate is a cellular metabolite with essential roles in the synthesis of glycosaminoglycans and proteoglycans and in cellular detoxification.

1.3 UDP-glucose dehydrogenase

Human UDP-glucose dehydrogenase (UGDH) is an enzyme that catalyzes the conversion of UDP-glucose to UDP-glucuronate by two successive oxidation reactions. Much has been learned about the structure, function and regulation of UDP-glucose dehydrogenase through *in vitro* kinetic assays, x-ray crystallography and *in vivo* investigations.

The initial characterizations of UGDH were performed using *S. pyogenes* and bovine liver UGDH, both enzymes that have a high similarity to human UGDH. These early studies revealed a number of important features of UGDH function. First of all, while UGDH was thought to exist as a homo-hexamer in humans and higher organisms, studies suggested that the hexamer contained only three active sites [16-18]. Also, it was recognized early on that a catalytic cysteine was essential for the reaction, though it was later realized that the cysteine is critical not in the first oxidation, but the second [19-22]. The roles of other active site residues have been clarified through mutagenesis, making possible the proposal of a reasonable reaction mechanism involving a first oxidation to a thiohemiacetal intermediate and

further oxidation to a thioester intermediate before release of the product by hydrolysis [8, 20, 22, 23].

The determination of the first crystal structure of UGDH, the *S. pyogenes* structure, informed many of the kinetic mutagenesis studies. The later availability of the *C. elegans* and human structures has confirmed the close structural similarity of the human enzyme to the bacterial enzyme. The available crystal structures highlight an interesting feature of the enzymes that has been demonstrated by other methods as well: the bacterial enzyme is a dimer, while the human enzyme is a hexamer.

In vivo studies of UGDH have focused primarily on regulation of the enzyme at the transcriptional and post-translational levels, as well as, the involvement of UGDH in epithelial cancers and development abnormalities. UGDH is known to be transcriptionally up regulated in response to androgens in various tissue types, including the prostate epithelium and breast cancer cells [24-26]. It has also been shown that the UGDH promoter contains a peroxisome proliferative receptor α (PPAR α) response element that mediates up regulation of UGDH in response to certain xenobiotics [27, 28]. At the post-translational level, UGDH activity responds to a variety compounds. UDP-xylose has long been recognized as an effective inhibitor of UGDH function [29]. A recent study also implicates two polyphenols, gallic acid and quercetin, in inhibition of UGDH activity in the cytosol [30].

UGDH has been associated with several pathological states in humans and

other species. The production of UDP-glucuronate for use in production of extracellular-matrix polysaccharides impacts the progression of many types of human epithelial cancers, including prostrate, breast, head and neck, ventricle, colon, and pancreas cancer [31]. Additionally, several recent studies in related organisms indicate UGDH has a significant, but poorly delineated, role in development. The effects may be severe and cause embryonic lethality as is the case when the *sugarless* gene of *D. melanogaster* contains certain mutations [11] or less severe as is the case of mutations of the *sqv-4* gene of *C. elegans* that results in defects in vulval morphogenesis [32-34]. Mutation of the *jekyll* gene, which encodes UDP-glucose dehydrogenase, of zebra fish results in defects in heart valve formation [35]. These phenotypic developmental problems seem to stem from problems with the formation of proteoglycans and glycosaminoglycans, which require adequate levels of UDP-glucuronate precursors.

In conclusion, UDP-glucose dehydrogenase is an important enzyme in human development and in the progression of many types of human epithelial cancers. Therefore, a more detailed understanding of the structure and function of this enzyme is essential. Recently, mutations in UGDH were identified that are associated with congenital heart defects. These mutations cause a change in oligomeric state of the enzyme from a hexamer to a dimer. This shift in oligomeric state may explain the observed phenotype. Therefore, understanding the differences in *in vitro* and *in vivo* activity and regulation between the wild-type hexameric enzyme and the mutant dimeric enzymes is essential to understanding the function of UGDH in development and may provide insight into the role of UGDH in progression of epithelial cancers.

1.4 Glutathione

Like UDP-glucuronate, glutathione is an essential cellular metabolite, not only because of its role in cellular detoxification, but also because of its significant role as a reductive agent in the oxidative stress response and maintenance of cellular redox homeostasis [5, 6, 36]. Glutathione is also important for cysteine storage and transport and in altering sulfhydryl accessibility to modulate signaling pathways and regulate enzyme activity [38, 49, 90].

Glutathione is a tripeptide composed of glutamate, cysteine, and glycine. It is synthesized by the sequential action of two enzymes, glutamate cysteine ligase and glutathione synthetase [76, 97]. In the first and rate-limiting step of glutathione biosynthesis, glutamate cysteine ligase combines cysteine and glycine to form □-glutamylcysteine in an ATP-dependent reaction [37]. Next, glutathione synthetase, also requiring ATP, adds glycine to form reduced glutathione [37, 38]. Oxidized glutathione is efficiently reduced by glutathione reductase, a flavoprotein that uses NADPH as a reductant, to maintain the cytosolic pool of glutathione in a reduced state [39].

1.5 Glutamate Cysteine Ligase

Glutamate cysteine ligase (GCL) is a cytosolic protein responsible for the first and rate-limiting step of glutathione biosynthesis. In humans and other higher eukaryotes, GCL is composed of heavy catalytic subunit (73 KD) and a light modifier subunit (30 KD) [40, 41]. The catalytic subunit is sufficient for catalysis, while the modifier subunit is known to enhance the activity of the catalytic subunit [44].

Additionally, the availability of the modifier subunit contributes to regulation of enzyme activity by limiting formation of the more active heterodimer. GCL is also regulated by feed-back inhibition by the down-stream product, glutathione [42]. Glutamate cysteine ligase has been widely characterized in a number of organisms. Characterizations of glutamate cysteine ligase have revealed much about both the complex reaction mechanism of the enzyme and the enzyme's *in vivo* functioning and regulation.

Three classes of GCL have been denominated based on sequence conservation: the □ -proteobacteria (group 1), the non-plant eukaryotes (group 2), and the plants and α-proteobacteria (group 3). However, functional characterizations and comparisons of the crystal structures of *E.coli* (Group1) and *B. juncea* (Group 3) with the recently published *S. cerevisiae* (group 2) structure have indicated that the reaction mechanism between groups is likely to be very similar [43-47]. Although the *S. cerevisiae* enzyme lacks a modifier subunit, it has 45% sequence identity with the human catalytic subunit [45]. Consequently, the *S. cerevisiae* structure has allowed for an accurate model of the human enzyme catalytic subunit to be generated, providing further insights into the catalytic mechanism.

The first step of the catalytic mechanism is an inline attack of the □-phosphate of ATP by the □-carboxylate of glutamate to form a □-glutamylphosphate intermediate [48]. Next, the α-amino group of cysteine performs a nucleophilic attack, releasing the phosphate and forming the product □-glutamylcysteine [49,

50]. The active site is particularly complex due to the presence of three bound magnesium ions and three substrates: glutamate, cysteine, and ATP. While debate exists over whether the substrates are ordered or enter the active site randomly, the examination of the human homology model based on the *S. cerevisiae* structure suggests, at least, that glutamate enters the active site before ATP and cysteine [44].

Glutathione biosynthesis is carefully controlled by regulation of GCL at multiple levels. At the post-translational level, glutathione acts as a feedback inhibitor, while cysteine availability controls the overall rate of the reaction [38, 42]. The association of the modifier subunit with the catalytic subunit has a significant impact on activity; mice without the modifier subunit produce approximately 10-20% the normal amount of glutathione [51]. Because GCLM availability limits heterodimer formation, increased expression of the modifier subunit may enhance GCL activity [51-54]. Heterodimer formation appears to be regulated by one or more non-essential disulfide bonds between the subunits and possibly by other post-translational modifications or inter-subunit interactions [55, 56].

GCL activity is also increased in response to oxidative stress without production of additional protein [51, 57, 58].

Additionally, glutamate cysteine ligase is subject to extensive transcriptional regulation. The human GCLM and GCLC transcripts are located on different chromosomes and are therefore subject to different regulation [51, 59, 60]. Furthermore, the levels of GCLM and GCLC vary widely in different human tissue types [61]. A number of transcriptional regulatory factors have been identified that

interact with GCL promoters. These include Nrf2, AP1, AP3, NFκB, Maf proteins, JunD, Fra, and CREB [62].

Not surprisingly, glutamate cysteine ligase has been associated with numerous diseases. Failure of glutathione production impacts the progression of many diseases, including HIV, cancer, cystic fibrosis, Alzheimer's disease and Parkinson's disease [63]. The importance of GCL to cellular homeostasis and human health is highlighted by the fact that GCLC null mice are embryonic lethal [64]. Interestingly, the absolute requirement for GCLC appears to be satisfied even in the heterozygous state when dramatically lower levels of glutathione are produced. Recently, GCLC mutations have been identified in individuals with hereditary glutathione deficiency, a disease characterized by hemolytic anemia and sometimes accompanied by neurological degeneration [65-69]. The erythrocyte glutathione levels in individuals homozygous for the mutations were >10% of normal levels. Because none of these mutations lie in the GCLC active site, it is not clear why they cause such a dramatic reduction in glutathione levels. Further studies of the mutations involved in this disease have the potential to lend significant insight into GCLC function. This work details our investigation of these GCLC mutants including assessment of glutathione production in GCLC null mouse embryonic fibroblast cells and the GCLC null *S. cerevisiae* ΔGSH1 strain, as well as, kinetic characterizations in the presence and absence of the modifier subunit.

Chapter 2

Effects of altered oligomeric state on UDP-glucose dehydrogenase function *in vitro* and *in vivo*

Note: The results described in this chapter are being prepared for publication and were completed by Melanie Neely Willis, Katie Easley, Alisha O' Malley, Joseph J. Barycki, Melanie A. Simpson, and members of Jeroen Bakker's laboratory.

2.1 Introduction

UDP-glucose dehydrogenase catalyzes the conversion of UDP-glucose to UDP-glucuronate via two successive oxidations, concomitantly converting two molecules of NAD^+ to NADH. UDP-glucuronate is an essential precursor for protein glycosylation, production of extracellular matrix polysaccharides and is necessary for cellular detoxification of xenobiotics and endobiotics via UDP-glucuronosyltransferases [8, 20]. Availability of UDP-glucuronate and UGDH expression have been implicated in a variety of epithelial cancers and in developmental abnormalities [70-76]. Therefore, a detailed understanding of the structure, function, and regulation of UGDH is essential.

While UDP-glucuronate is implicated in a variety of cellular processes, recent advances in understanding both developmental abnormalities and cancer progression associated with UGDH have highlighted the role of hyaluronan, a key extracellular matrix polysaccharide in causation of both processes [75]. The common link between development and cancer progression is hyaluronan's promotion of cell migration and proliferation. While the types of cancer associated with increased hyaluronan production include breast, head and neck, ventricle, colon, and pancreas cancer [31]; hyaluronan and UGDH have received significant attention in prostate cancer progression, and UGDH has been named as a potential biomarker for prostate cancer [77]. While much has already learned about UGDH, these advances demonstrate the necessity of fully delineating the many factors that control UGDH function in the cell.

Detailed kinetic characterizations and mutation of key catalytic residues by our lab and other groups have illuminated key steps in the reaction mechanism of UGDH. These studies have been greatly aided by modeling the human enzyme using the available *S. pyogenes* crystal structure (PDB ID: 1DLJ) and later, the *C. elegans* (PDB ID: 2O3J) and human crystal structures (PDB ID: 2QE3). Early studies of the UGDH catalytic mechanism were made using *S. pyogenes* and bovine liver enzyme. A Bi-Uni-Uni-Bi Ping Pong mechanism was proposed in which the mechanism proceeded through an UDP-aldehyde intermediate that was trapped by an active site lysine to form a Schiff base intermediate [19, 78, 79]. An active site cysteine would then attack the Schiff base, and the reaction would proceed to completion through a thiohemiacetal intermediate, followed by further oxidation to a thioester intermediate and hydrolysis to yield the product. Ultimately, the formation of a Schiff base has been discredited [20, 23]. Kinetic data from our lab suggest that in the human enzyme, K270 serves to position D280 in the active site, which, in turn positions and activates a catalytic water molecule. The water molecule may then directly abstract a proton from the 6' hydroxyl of UDP-glucose in the first oxidation step and be repolarized by K220. The repolarized water might then activate the catalytic cysteine, C276, allowing attack to form the thiohemiacetal intermediate. The K220 residue could then serve to stabilize the oxyanion charge during formation of the thioester intermediate and hydrolysis to yield the product, UDP-glucuronate [20, 22].

In recent years, the knowledge of UDP-glucose dehydrogenase regulation *in vivo* has rapidly advanced fueled, in part, by the association of UGDH with various

cancers and developmental aberrations. Several studies have reported that the UGDH gene is under the control of the peroxisome proliferative receptor α (PPAR α) response element, allowing an up-regulation of UGDH in response to some xenobiotics [27, 28]. Similarly, UGDH has been demonstrated to be up-regulated at the transcriptional level in response to androgens in certain cancers [24-26]. These findings are not surprising considering the key role UGDH plays in detoxification and epithelial cancer progression, and future studies will likely expand and confirm the role of transcriptional regulation in UGDH function and malfunction.

UGDH is also subject to extensive post-translational regulation, including inhibition by UDP-xylose, quercetin, and gallic acid [9, 20, 21]. UDP-xylose is produced from UDP-glucuronate in the ER and Golgi lumen, making it a potent feedback inhibitor [Bakker]. Quercetin and gallic acid are both members of the class of compounds known as polyphenols that have recently been linked with prostate cancer tumor suppression [21]. While quercetin and UDP-xylose have been proposed to be competitive inhibitors of UDP-glucose in the enzyme active site, gallic acid was found to be a non-competitive inhibitor [9, 21]. Additionally, UDP-xylose was shown to have effects on allosterism, and quercetin was found to have mixed-type inhibition with respect to NAD^+ [20, 21]. A large-scale screen of chemicals by our lab revealed a number of novel inhibitors that have yet to be characterized.

Thus, it is possible that post-translational regulation of UGDH may proceed by binding or interaction with sites on the surface of the protein, distant from the enzyme active site. This possibility is significant because bacterial forms of UGDH

are dimeric, while human and other higher species have a hexameric structure, a trimer of active dimers (Figure 2.1). In the human enzyme, regulation may occur at sites not found in the dimeric enzyme, such as at the dimer-dimer interface.

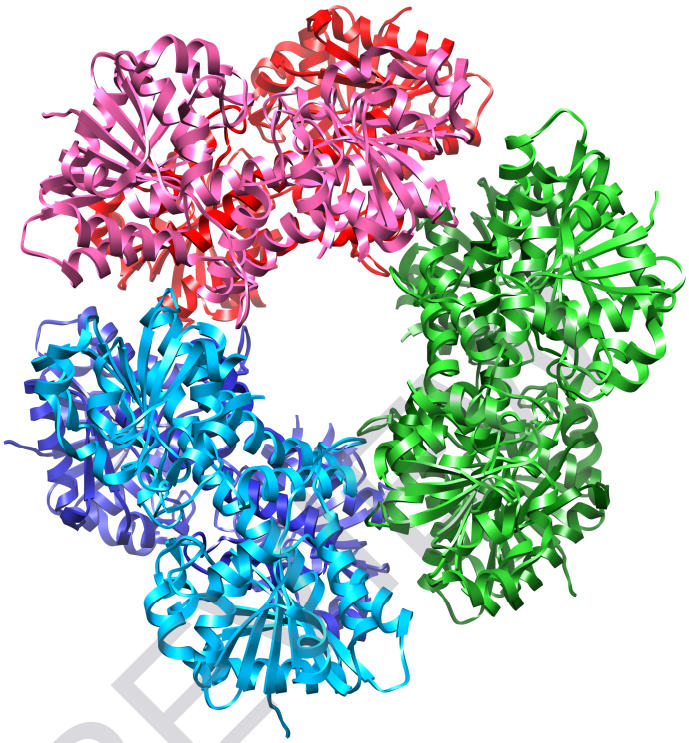

Figure 2.1. The human UDP-glucose dehydrogenase is a homohexamer. In humans and other vertebrates, UGDH exists as a hexamer, a trimer of catalytically active dimers. Here one dimer pair is shown in pink/red, one in blue/cyan and one in dark green/light green. PDB ID: 2Q2E.

Furthermore, recent studies by our collaborator, Jeroen Bakker, have revealed that certain mutations in human UGDH are associated with congenital heart defects [80]. Further studies of these two mutants, UGDH E416D and UGDH R141C, confirmed the link between these mutations and aberrant cardiac development in zebra fish. Knockdown of UGDH using a morpholino in zebra fish embryos caused a dramatic increase in cardiac edema. Yet, this phenotype was almost wholly abolished by addition of wild-type UGDH RNA. Partial rescue was observed when either UGDH E416D or UGDH R141C RNA was added (Figure 2.2), indicating that

there are *in vivo* differences in the functioning of these two mutant UGDHs compared to wild-type.

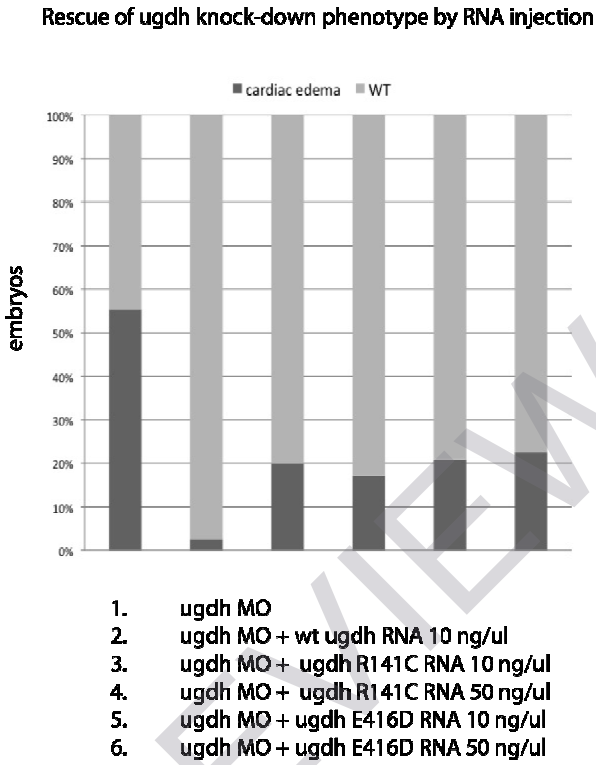

Figure 2.1. Rescue of UGDH knockdown phenotype by RNA injection. Shown in the above figure is the percentage of embryos developing an aberrant phenotype of cardiac edema (dark grey bar) versus a wild-type phenotype (light grey bar) for each population. The different populations are enumerated and correspond to each bar from left to right and indicate the type of treatment. All embryos contain an UGDH morpholino to knockdown UGDH expression. Selected populations were injected with human wild-type or UGDH E416D or UGDH R141C RNA with a concentration of either 10 ng/µl or 50 ng/µl.

Although these two mutations have a demonstrated phenotype in zebra fish embryos, examination of the human crystal structure of UGDH reveals that these two mutations are neither located in, nor are very close to the enzyme active site. This observation raises the intriguing possibility that rather than directly altering enzyme catalysis, these mutations may be important to enzymatic regulation.