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REVIEW

Evolution of urea transporters in vertebrates: adaptation to urea's multiple roles and metabolic sources

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ABSTRACT

In the two decades since the first cloning of the mammalian kidney urea transporter (UT-A), UT genes have been identified in a plethora of organisms, ranging from single-celled bacteria to metazoans. In this review, focusing mainly on vertebrates, we first reiterate the multiple catabolic and anabolic pathways that produce urea, then we reconstruct the phylogenetic history of UTs, and finally we examine the tissue distribution of UTs in selected vertebrate species. Our analysis reveals that from an ancestral UT, three homologues evolved in piscine lineages (UT-A, UT-C and UT-D), followed by a subsequent reduction to a single UT-A in lobe-finned fish and amphibians. A later internal tandem duplication of UT-A occurred in the amniote lineage (UT-A1), followed by a second tandem duplication in mammals to give rise to UT-B. While the expected UT expression is evident in excretory and osmoregulatory tissues in ureotelic taxa, UTs are also expressed ubiquitously in non-ureotelic taxa, and in tissues without a complete ornithine–urea cycle (OUC). We posit that non-OUC production of urea from arginine by arginase, an important pathway to generate ornithine for synthesis of molecules such as polyamines for highly proliferative tissues (e.g. testis, embryos), and neurotransmitters such as glutamate for neural tissues, is an important evolutionary driving force for the expression of UTs in these taxa and tissues.

KEY WORDS: SLC14, Arginine, UT evolution

Introduction

The ability of animals to translocate urea across internal membranes as well as across epithelia in contact with the environment has been known for decades. In many early models, urea movement was viewed as occurring directly across the lipid bilayer. Many physiological phenomena seemed in conflict with this early paradigm, notably the remarkable urea gradients set up in the mammalian kidney (see below). Thus, it was perhaps not surprising when You et al. (1993) described the cloning of the first urea transporter (UT) from the rabbit kidney, notably through the labour-intensive method of expression cloning. In the ensuing two decades, urea transporters have been identified from all manner of species from microbes to man. Interestingly, however, much of the focus on the UTs has been in the context of urea excretion to the environment by 'ureoteles' (animals excreting the majority of their nitrogenous waste as urea). However, as pointed out in some prior reviews (e.g. see Withers, 1998) and reiterated and re-examined below, there are many metabolic paths to the urea molecule, and many physiological functions for urea. Thus, this review aims to summarize the now substantial sequence information for UTs from many taxa (with special focus on vertebrates) and to examine distributions of UT homologs in the light of these multiple roles for urea.

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Urea: metabolic pathways and physiological roles

Before we discuss the structural and functional evolution of UTs in vertebrates, a brief discussion of the metabolic pathways generating urea and its physiological significance in vertebrates is necessary. This is described in more detail elsewhere (see Walsh and Mommsen, 2001; Withers, 1998; Wright, 2007); here, we will summarize our current understanding of ureogenesis and its functional value in vertebrates.

Metabolic aspects of ureogenesis

Urea is a nitrogenous compound that was discovered almost 300 years ago, and was the first organic compound to be synthesized just a century later (see Kurzer and Sanderson, 1956, for a historical perspective). When examined at the metabolic level, urea can be produced through a number of catabolic and anabolic pathways. Although the exact contribution of each individual pathway is still under investigation, particularly in non-mammalian taxa, we present below a general introduction to the ureogenic metabolic pathways.

The ornithine–urea cycle

In ureotelic vertebrates, the primary metabolic pathway involved in urea production is the ornithine–urea cycle (OUC) (Fig. 1). This anabolic pathway allows ammonia to be detoxified into urea through a series of mitochondrial and cytosolic enzymes. An important evolutionary aspect of this pathway is that it has clearly evolved from ancestral metabolic pathways that are common to virtually all organisms. Indeed the OUC is composed of two linear reactions, arginine synthesis [via carbamoyl phosphate synthetase I/III (CPSI/III), ornithine transcarboxylase, argininosuccinate synthase and lyase] and arginine degradation through the enzyme arginase (Fig. 1). One of the main evolutionary innovations leading to the acquisition of the OUC is the duplication of the cytosolic enzyme CPSII, and its translocation to the mitochondria, becoming CPSI (in tetrapods) or III (in fishes). This pathway was initially thought to be a metazoan invention; however, recent advances in comparative genomics suggest that the evolution of a mitochondrial CPS predates metazoans as it is present in choanoflagellates as well as in a number of diatom species (Allen et al., 2011). However, this trait was probably secondarily lost in several metazoan lineages, and the investigation of its physiological significance in primitive eukaryotes will certainly prove to be an active field of study in the near future.

Purine degradation and uricolysis

In amphibians and fishes, urea can also be generated through the degradation of purines and uric acid (uricolysis). This pathway, catabolic in nature, involves a series of oxidases and hydrolases

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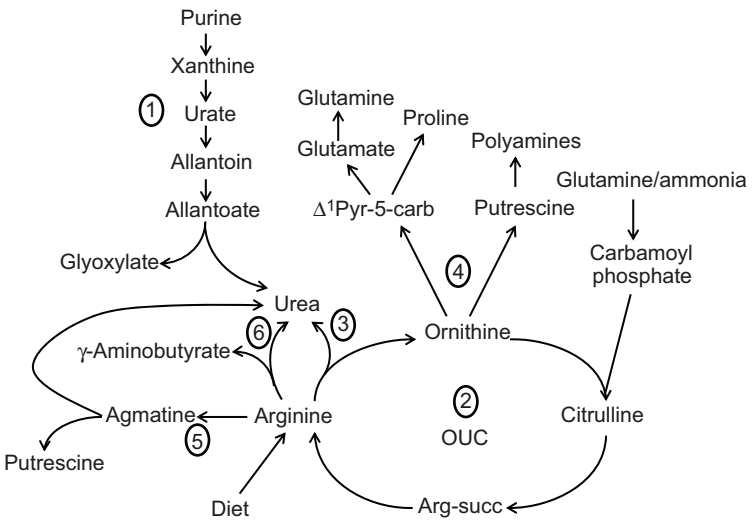

Fig. 1. The multiple metabolic pathways producing urea. (1) The purine degradation pathway is composed of xanthine oxidase, urate oxidase, allantoinase and allantoicase, generating glyoxylate and urea. (2) The ornithine–urea cycle (OUC) uses either ammonia (tetrapods) or glutamine (fish) as substrates to first synthesize arginine via carbamoyl phosphate synthase (CPS)I/III, ornithine–carbamoyl transferase, argininosuccinate synthase and lyase (Arg-succ, argininosuccinate); the argininolytic part of the cycle, via arginase (3), produces urea and ornithine. (4) The ornithine generated via the OUC or alternative routes can be diverted into polyamine production (via ornithine decarboxylase, spermine and spermidine synthethase), or first into Δ^1pyrroline-5-carboxylate (Δ^1Pyr-5-carb) through ornithine δ-aminotransferase and then further processed into proline (by Δ^1Pyr-5-carb reductase) or into glutamate and glutamine (Δ^1Pyr-5-carb oxidase and glutamine synthetase). (5) The agmatine pathway yields putrescine and urea via arginine decarboxylase and agmatine ureohydrolase. (6) The γ-aminobutyrate pathway also generates urea through the oxidative deamination of arginine, 2-oxo-guanidinovalerate decarboxylase and γ-guanidinobutyrate urea hydrolase. Adapted from Walsh and Mommsen (2001).

catalysing the conversion of guanine and adenine to uric acid, and further hydrolysis of uric acid into urea (Fig. 1). Although this pathway was thought to be negligible in the nitrogen budget of vertebrates, several lines of evidence suggest that uricolysis may represent a relatively important contribution to nitrogenous waste excretion and urea production in ammonotelic vertebrates (Andersen et al., 2006; Terjesen et al., 2001; Wilkie et al., 1999; Wright, 1993).

Arginine catabolism

Arginine is a primary substrate (argininolysis) or intermediate (OUC, see above) for the majority of the metabolic pathways generating urea in vertebrates (see Walsh and Mommsen, 2001). Arginine is considered an essential amino acid, particularly during periods of rapid cellular proliferation and early development. Whether it is endogenously produced or obtained from the diet, arginine can be metabolized into urea via a number of enzymes. Central to argininolysis and abundantly expressed in a variety of vertebrate tissues, arginase catabolizes arginine into urea and ornithine. In turn, ornithine is a crucial intermediate in the synthesis of proline, glutamate and glutamine, putrescine and polyamines as well as citrulline (Fig. 1). Alternatively, the γ-guanidino urea hydrolase pathway can generate urea as a by-product of γ-aminobutyrate synthesis. The agmatine pathway is another alternative pathway to arginase that can also generate urea and putrescine from arginine.

Functional role of urea in vertebrates

Besides the purely mechanistic consideration of ureogenesis, it is important to recognize the multiple roles that urea plays in organisms. Our discussion of the subject is only meant to briefly summarize the many physiological roles of urea across a variety of taxa, and we direct the reader to more exhaustive review articles on this theme for more detailed information (e.g. Withers, 1998).

Nitrogen source

Urea is an important nitrogen source in several prokaryote species; whether intracellularly produced or harvested from the environment, it is an essential point in the nitrogen utilization pathway in these organisms (Alonso-Sáez et al., 2012; Solomon et al., 2010). An interesting example is the process of urea nitrogen salvaging in the guts of several mammalian species through symbiotic interactions with a variety of ureolytic microorganisms (reviewed in detail in Stewart and Smith, 2005). These bacteria can harvest and degrade gastrointestinal urea produced from their mammalian hosts and use the nitrogen as a building block for amino acid and protein biosynthesis. The bacterial products can then be transported across the gastrointestinal epithelium of the mammalian host, thereby allowing the host to re-utilize the nitrogen from the urea it initially generated (Stewart and Smith, 2005). In other taxa, such as the photosynthetic marine diatom *Phaedactylum tricornutum*, urea is also a crucial component of nitrogen utilization, playing a predominant role in carbon fixation into nitrogenous compounds (via an active OUC), the formation of cell walls, as well as in the production of numerous osmolytes (Allen et al., 2011).

Osmolyte

Urea is an essential balancing osmolyte in a number of marine vertebrates (Griffith, 1991; Withers et al., 1994; Yancey et al., 1982). In particular, cartilaginous fishes and the coelacanth are virtually iso-osmotic to seawater and typically maintain a very high urea concentration to preserve homeostasis. A number of other vertebrates also use urea as an osmolyte in conditions of osmotic stress. For example, several amphibians and fish species produce and retain urea at higher concentration when faced with adverse conditions, such as increased salinity or dehydration, thereby maintaining homeostasis by preventing excessive water loss (Abe and Bicudo, 1991; Gordon and Tucker, 1968; Jørgensen, 1997; Romspert and McClanaham, 1981; Saha and Ratha, 1989, 1994).

Cryoprotectant and metabolic regulator

Although amphibians accumulate urea in their tissues when subjected to osmotic stress (see above), more recently a novel role for urea was uncovered in the freeze-tolerant wood frogs: cryoprotection (Costanzo and Lee, 2005, 2008; Costanzo et al., 2008). Hibernating frogs are able to accumulate urea in their tissues and plasma (Schiller et al., 2008), a strategy that protects their cells and tissues against the noxious effects of freezing and thawing, including iono-osmotic stress and mechanical injuries (Costanzo and Lee, 2005, 2008; Costanzo et al., 2008). Urea may also promote a hypometabolic state in hibernating ectotherms via destabilization of proteins and enzymes (Muir et al., 2008). Moreover, urea loading in these frogs is associated with faster neurobehavioural recovery rates post-thawing, demonstrating that urea not only has a beneficial effect at the tissue level but also confers an important adaptive advantage for the whole organism (Constanzo and Lee, 2008). Notably, the review by Yancey and Siebenaller (2015) in this issue

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discusses the de-stabilizing effects on proteins of the high urea levels achieved in animals such as elasmobranchs and freeze-tolerant amphibians, and the evolutionary solutions to counteract these effects.

Urine concentration

The role of urea and the general anatomy of the mammalian kidney in urine-concentrating mechanisms has been under extensive investigation over the last few decades, and has been recently reviewed in great detail (see Pannabecker, 2013; Yang and Bankir, 2005). In brief, the mammalian kidney concentrates urine to an exceptional degree, primarily due to a countercurrent multiplication of NaCl, the capacity of which is greatly enhanced by urea gradients. These urea gradients are predominantly possible because of the particular architecture of the kidney as well as the differential urea permeability of the different sections, allowing not only osmotic retention of water and concentration of urine but also urea recycling within the kidney.

Nitrogenous waste

Urea is also a predominant nitrogenous waste in a number of organisms regardless of their osmoregulatory strategy. As described above, generating urea in the OUC allows for the detoxification of noxious ammonia produced by amino acid catabolism. This mechanism is of particular importance in animals that do not have the luxury of directly excreting ammonia into their immediate environment. Particularly, aquatic animals that cannot simply excrete ammonia into the water because of physical or chemical limitations rely heavily on the OUC and urea production to avoid hyperammonaemia. One example is the developing teleost embryo, which during the initial phase of development is enclosed in a semi-permeable chorion that limits the direct loss of ammonia to the surrounding water. In zebrafish and trout embryos, the OUC is active and physiologically relevant during the early stages of development, and when knocked down, significant levels of ammonia accumulate within the egg (LeMoine and Walsh, 2013; Wright et al., 1995). However, the OUC is expensive (5 mol of ATP are required in fish to synthesize urea), so these pathways are silenced near the time of hatching. Another classical example is the Lake Magadi tilapia (*Alcolapia grahami*), that remains obligately ureotelic through adulthood, a rare feat in teleosts (Randall et al., 1989). These fish live in a very alkaline and highly buffered lake in Kenya, an environment that physically prevents the excretion of catabolically produced ammonia into the surrounding water. In place of ammonia, these fish excrete the majority of their nitrogenous waste as urea as a result of high hepatic and muscular OUC capacities (Lindley et al., 1999; Randall et al., 1989).

Chemical cloak

In at least one ureotelic teleost, the Gulf toadfish (*Opsanus beta*), the use of urea as an excretory product is thought to provide a chemical cloak against predators (Barimo and Walsh, 2006). The toadfish, like the Lake Magadi tilapia, is one of the few teleost species that remains ureotelic throughout its lifecycle (Mommsen and Walsh, 1989; Barimo et al., 2004). However, unlike most ureoteles, toadfish in mesocosms excrete the majority of their urea and ammonia at a 1:1 ratio in distinct diurnal pulses (Barimo et al., 2010). In addition, a common predator of the toadfish, the grey snapper (*Lutjanus griseus*), readily detects low ammonia concentration in these microcosms, but is less likely to display aggressive behaviour when exposed to either urea alone or urea and ammonia combined (Barimo and Walsh, 2006). Thus, in these animals, adult retention of ureotely may be adaptive and provide predator avoidance, but this phenomenon remains to be explored in other ureotelic fishes.

Structure and genomics of UTs

The UT proteins belong to the solute carrier family of transporters (SLC), a large family of proteins that transports substances such as carbohydrates, nucleotides, amino acids, ions and various chemicals across cell membranes. In particular, the UTs (SLC14A) allow the facilitated diffusion of urea across biological membranes. Although the body of work on mammalian systems has been recently reviewed in greater detail (see Shayakul et al., 2013), in the following sections we integrate this information with data obtained from non-mammalian taxa to provide an overview of the current knowledge of UT structure, gene organization and subcellular localization in vertebrates.

UT structure

The predicted primary structure of the majority of UT proteins is composed of 10 transmembrane domains, with two internal hydrophobic halves connected by an extracellular loop (You et al., 1993). Although the predicted transmembrane domain and overall structure may differ slightly across taxa, the relatively high conservation of the homologous transporters across vertebrates suggests a similar overall architecture (Minocha et al., 2003). In mammals, the intracellular amino-terminus and extracellular carboxy-terminus harbour multiple post-translational regulatory sites, including N-glycosylation and phosphorylation sites (Olives et al., 1994; You et al., 1993). Although not empirically tested, our *in silico* analyses reveal that most metazoan UTs also have a number of potential kinase-regulated phosphorylation sites in their termini.

Recently, two groups reported the high resolution crystal structure of bacterial homologues of the mammalian UT-A (Levin et al., 2009; Raunser et al., 2009). Interestingly, in one bacterial species (*Desulfovibrio vulgaris*), the transporter functions as a trimer, but it was determined to have a dimeric structure in another species (*Actinobacillys pleuropneumoniae*). More recently, Levin and colleagues resolved the homotrimeric structure of the bovine UT-B further, begging the question of whether different UT homologues have different multimeric structures across and within species (Levin et al., 2009, 2012; Raunser et al., 2009). However, given the complexity of the gene structure in metazoans, including humans (see below), the multimeric structure of the different UT isoforms in metazoans remains elusive and has yet to be fully characterized.

UT genes

In mammals, UT-A and UT-B are encoded by two distinct genes located on the same chromosome (chromosome 18 in humans), each giving rise to multiple alternatively spliced isoforms, at least six for UT-A and two for UT-B (Sands, 2003). Although in most other vertebrate species investigated here the different UT genes are all located on distinct chromosomes (supplementary material Table S1), they do seem to share the potential for alternative splicing. For example, in elephantfish (*Callorhinchus milii*; Kakumura et al., 2009) and stickleback (*Gasterosteus aculeatus*), multiple predicted transcripts also exist, suggesting that the modularity of the UT gene transcripts is a recurring theme in vertebrates, and adding to the complexity of characterizing their structure and function.

The mammalian UT-A gene contains 20–24 exons giving rise to proteins of 43–120 kDa (see Sands, 2003). Similarly, the avian

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UT-A spans 20–22 exons and its predicted product has a molecular mass similar to mammalian UT-A. The mammalian UT-B gene is encoded by 11 exons and is translated into proteins of approximately 43–45 kDa. In amphibians, the coelacanth and other fishes, UT genomic organization is slightly variable, but overall the proteins are encoded by 7–10 exons, translating into a predicted 40–45 kDa polypeptide.

Subcellular localization

Within the cell, the UT proteins investigated to date primarily localize to the cell membrane or are enclosed within cytoplasmic vesicles (Bucking et al., 2013a,b; Nielsen et al., 1996; Fenton and Knepper, 2007). These vesicles probably play an important role in the cellular trafficking, storage and recycling of the UT depending on the cellular need for urea transport. In addition, there have been suggestions that some non-mammalian species may harbour facilitated urea transporters within their mitochondrial membrane, a feat that could be of physiological importance in species that produce urea within the organelle (Rodela et al., 2008). Our analysis of the sequences for all the UTs currently available suggests that at least a few species from diverse taxa potentially have a mitochondrial leader sequence signal that could allow translocation of the transporters into the mitochondria: the diatom (aUT-2), elephantfish (UT-D1), stickleback (UT-C) and platyfish (UT-D2). This is not to say that other species, particularly other teleosts and cartilaginous fishes having relatively poor genomic coverage, do not possess any UT isoforms that could be mitochondrially bound, but it is clear that no tetrapods have this capacity.

UT evolution

The current nomenclature of the UT family is a legacy of the initial discovery of these proteins in mammals, and subsequent discoveries in other taxa have complicated the proper identification of each protein according to its phylogenetic history (Mistry et al., 2005; Olives et al., 1994; You et al., 1993). Owing to recent advances in the field of comparative genomics and the availability of molecular sequences from a variety of taxa, we have generated a phylogenetic reconstruction of the UT family (Fig. 2). Further, taking this evolutionary history into consideration, we propose several hypotheses regarding the driving forces behind major evolutionary innovations in the UT family.

UT phylogeny

Our phylogenetic reconstruction of the UT family suggests that these transporters have a dynamic history throughout vertebrate evolution (Fig. 2). The presence of a single UT gene in prokaryotes and the majority of basal eukaryotes suggests that the whole UT family evolved from a single ancestral gene, which for simplicity we will call aUT. This single gene was further conserved in primitive metazoans and in protochordates. It should be noted that in at least one primitive metazoan (a marine diatom), aUT was duplicated. Nevertheless, the aUT gene was then repeatedly duplicated in fishes, once to give rise to UT-C in teleosts and chimaeras (previously UT-γ in elephantfish), and a potential secondary lineage-specific duplication of the UT in chimaeras and acanthomorph teleosts giving rise to a third UT in these species (Fig. 2). For simplicity, and to keep with the current nomenclature (UT-A, -B and -C), we propose to name these isoforms UT-D1 in chimaeras and UT-D2 in teleosts. These UT-D isoforms exhibit on average much higher amino acid identity to UT-A (56% in teleosts, 61% in elephantfish) than UT-C (37% in teleosts, 50% in elephantfish), suggesting that they are probably derived from UT-A duplications. This phylogenetic signal is corroborated by the presence of all three genes on three independent chromosomes in the three teleosts for which genomic coverage is sufficient (platyfish, medaka, stickleback; see supplementary material Table S1). In addition, the synteny of the secondarily derived fish UT-D2 is highly homologous to the fish UT-A, suggesting a more recent duplication of that gene when compared with UT-C, which would have had more evolutionary time to diverge in terms of both gene sequence and chromosomal arrangement.

The presence of a single UT in primitive lobe-finned fishes, the lungfish and coelacanth, suggests that the previously duplicated UT isoforms were lost prior to the Sarcopterygian radiation. Further, the presence of a single homologous UT gene in amphibians supports the early deletion or pseudogenization of duplicated UT genes in primitive tetrapod lineages. A relic of this gene reduction is still evident in the genome of some mammals, such as in mouse, where a UT pseudogene can be found in a genomic location distinct from the two functional UT isoforms (chromosome 4 versus chromosome 18). In amniotes, the UT family undergoes two major innovations. First, a trait shared by mammals, birds, an alligator and a turtle species, is the internal duplication of the lobe-finned fish primitive UT, giving rise to an architecture similar to the modern UT-A gene of mammals. This internal duplication may not have been retained in other vertebrate lineages (i.e. squamates), although the paucity of molecular and genomic information on these species does not allow us to claim it with absolute certainty. The second major innovation is the tandem duplication of the UT gene in mammals giving rise to UT-B. In humans, this second UT gene is located a mere 50 kb away from UT-A and displays a high degree of homology to its paralogue (Fenton et al., 2002). This peculiar series of duplication/reduction/duplication that gave rise to the modern UT topology in vertebrates generates a number of evolutionary and physiological questions.

Evolutionary and physiological implications

First, it is now clear that a number of large duplication events occurred at multiple points in vertebrate history (Ohno, 1970; Van de Peer et al., 2009). Notably, two large duplication events are thought to have happened prior to the diversification of cartilaginous fishes (sharks, rays) and at least one occurred within the teleost lineage (Meyer and Van de Peer, 2005; Van de Peer et al., 2009). Taking that into account, the expansion of the UT family in cartilaginous fishes and teleosts may not seem particularly surprising. However, the most common fate of a gene duplicate is pseudogenization or complete deletion (Zhang, 2003); thus, it is interesting to see remnants of these duplications expressed in these lineages, particularly when taking into consideration the presumed physiological roles of the UT in ammonotelic fish, for example.

Conversely, the evolutionary pressure that reduced the number of UT genes in early tetrapods is equally intriguing. Physiologically speaking, both the lungfish and the coelacanth are ureogenic, and urea conservation in the coelacanth may be of prime importance given the metabolic cost of producing urea and its use as an osmolyte in this fish (Pickford and Grant, 1967; Brown and Brown, 1967). Thus, it is possible that this reduction in the number and presumably a restriction of tissue distribution of UTs may have been an advantage to prevent excessive loss of urea to the environment in early Sarcopterygians, particularly from relatively permeable tissues like the gills.

Third, the internal duplication that gave rise to the modern UT-A in amniotes presents an interesting evolutionary step in the UT family, and is in part responsible for the diversity of UT-A

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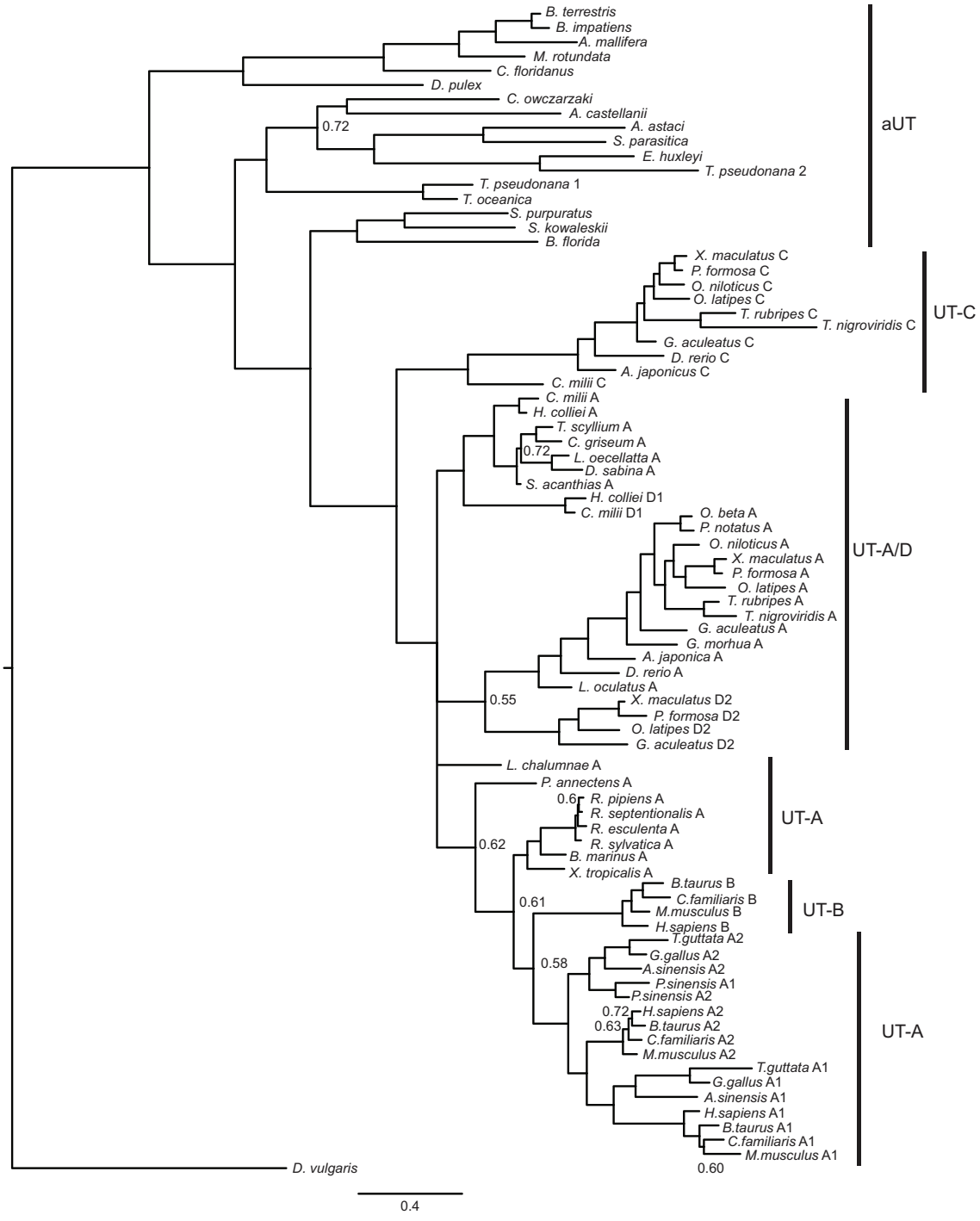

Fig. 2. UT phylogeny. The protein sequences for UT homologues were retrieved from public databases (Ensembl, GenBank; see supplementary material Table S1). In amniote UT-A, the two halves of the protein, the amino- (A1) and carboxy-termini (A2) were analyzed independently to provide comparable protein lengths across taxa. The sequences were aligned (ClustalX; Larkin et al., 2007) and a phylogeny of the family was reconstructed through Bayesian analysis (MrBayes v3.1.2; Ronquist and Huelsenbeck, 2003). The analysis was run using a fixed-rate model (PRSET) for 10^6 generations, sampling every 100 with 3 heated chains and one cold chain for each run, and a burn-in of 15% of the trees sampled (LeMoine et al., 2008). Except where noted, all nodes were supported by >75% posterior probabilities. The scale represents the mean number of substitutions per site.

alternative transcripts in mammalian species. The amniote radiation is associated with multiple physiological innovations that permitted the colonization of a terrestrial environment. Particularly, the fundamental change in reproductive strategy and the development of a cleidoic egg were important contributors to the success of this vertebrate group on land. The novelty of the amniote egg is the presence of three extraembryonic compartments surrounding the embryo, the amnios, chorion and allantois, that not only provide an aqueous environment for the embryo but also mediate between the embryo and the external environment. Indeed, in oviparous species, these compartments and their membranes are crucial for gas exchange, acid–base balance, water and electrolyte absorption as

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well as urinary waste product disposal. Of particular interest to our current discussion is the excretion of nitrogenous waste products. Most vertebrate embryos go through a ureogenic phase during early development (see discussion below) and of interest is that, regardless of the species examined (sharks, teleosts, birds, reptiles, mammals), extraembryonic structures (the allantois or the yolk sac) accumulate urea at higher concentration than the embryo proper. These observations suggest that mechanisms must be in place to allow for not only the extrusion of urea from the embryo but also its segregation within other structures. Thus, it is conceivable that a more complex anatomical structure of the embryo may have prompted a necessity for diversification of the UT allowing proper segregation of urea outside of the amniote embryo.

Finally, the appearance of a second UT in mammals is an important innovation in mammalian evolution. This second UT isoform, UT-B, was initially isolated from human erythropoietic cells (Olives et al., 1994). Beyond its relatively ubiquitous tissue distribution, it is expressed and functional in red blood cells and in the vasa recta of the kidney, in which it plays a major role in maintaining the efficiency of the countercurrent exchange in mammalian kidneys (Yang et al., 2002). Indeed, UT-B knockout mice have an impaired capacity to concentrate their urine, suggesting that the evolution of this transporter in mammals was a major evolutionary innovation allowing this ureotelic lineage to efficiently conserve water in a terrestrial environment (Yang et al., 2002).

Tissue distribution of UTs in vertebrates

The expression patterns of UT isoforms have been explored in a number of taxa, with an important bias towards tetrapods, particularly mammals. To gain a better understanding of the physiological role of the different vertebrate UT homologues, we examined their tissue distribution in various vertebrate species by RT-PCR and compiled these experimental results with gene expression patterns derived both from literature and public databases (Table 1). While in a number of tissues the presence of UT mRNAs was in line with physiological function (ureogenic, excretory or osmoregulatory tissue), we were particularly surprised to find a fairly ubiquitous presence of UTs in multiple tissues not traditionally associated with urea production.

Excretory and osmoregulatory tissues

Most animals produce at least modest amounts of urea that needs to be excreted; thus, it is not particularly surprising that excretory organs show UT expression. In vertebrates, the kidney is a major excretory tissue and at least one UT isoform is present across species. In mammals, the kidney also plays an important role in urea recycling and as such a number of distinct UT isoforms have a differentiated spatial expression in this organ. Another interesting observation is the fact that in toadfish renal cells, the primary UT expressed is UT-D2, whereas UT-A and C are expressed in the zebrafish kidney. It would be interesting to test the possibility that UT-D2 presence in toadfish plays a role in its capacity to excrete urea in a concentrated urine while other species, like the rainbow trout, do not have this capacity and appear to reabsorb urea rather than secrete it (McDonald and Wood, 1998; McDonald et al., 2000). Furthermore, it should also be kept in mind that in most vertebrates, the kidneys exhibit argininolytic and polyamine synthetic capacities (Jenkinson et al., 1996; Manen et al., 1976), which in turn may generate urea that would need to be exported out of renal cells via the UT. The gill of fishes is also an important site of excretion, so the presence of UTs in the gills of teleosts would presumably allow fish to excrete their urea waste through this epithelium (Bucking et al., 2013a,b; Walsh et al., 2000). Of interest, none of the cartilaginous fish investigated to date express a UT in their gills, in accordance with the relatively low branchial urea permeability (Boylan, 1967; Smith and Wright, 1999).

Ureogenic tissues

In mammals and several ureotelic vertebrates (e.g. dogfish, toadfish), the liver is the main ureogenic organ (Anderson, 1991; Brown and Cohen, 1960; Mommsen and Walsh, 1989); therefore, the presence of a hepatic UT is warranted in these species, though the functionality of the UTs in vertebrate hepatocytes has only been directly tested in a few instances (e.g. Jelen et al., 2012). In addition, in most vertebrates the liver is also a major site of arginine metabolism, and usually exhibits high levels of arginase enzymatic activity and polyamine synthetic capacity (Corti et al., 1987; Manen et al., 1976); thus, it is conceivable that the presence of UTs in the liver of most vertebrates, including non-ureotelic organisms, is also related to arginine metabolism. Similarly, UTs are ubiquituously expressed in skeletal muscle tissues (Table 1). This pattern may also be a reflection of the muscle ureogenic capacity in some fish species owing to the presence of OUC enzymes in their skeletal muscles (Kong et al., 1998; Korte et al., 1997; Lindley et al., 1999). Further, similar to the situation in liver, arginine metabolism and polyamine synthesis are essential to muscle proliferation and growth (see Mommsen, 2001), which in turn may necessitate the presence of UTs to extrude urea produced as a by-product of these pathways in muscles.

Table 1. Tissue distribution of UT in vertebrates

	Species						
Tissue	Zebrafish	Toadfish	Dogfish	Elephantfish*	Frogs‡	Birds§	Mammals¶
Gills/lungs	UT-A	UT-A	–	–	UTA	UTA	UT-B
Kidney	UT-C/A	UT-D2	UT-A	UT-A/C/D1	UTA	UTA	UT-A/B
Liver	UT-A	UT-D2	–	UT-A	UTA	UTA	UT-A/B
Intestine	UT-A	UT-A	UT-A	UT-D1	UTA	UTA	UT-B
Testis	UT-C	UT-C	UT-A	UT-D1	UTA	UTA	UT-B/A
Brain	UT-C/A	UT-C	UT-A	UT-D1/A	UTA	UTA	UT-B
Heart	UT-A	UT-A/D2	UT-A	UT-D1	–	UTA	UT-A/B
Skeletal muscle	UT-C/A	UT-A/D2	UT-A	UT-A/D1	–	UTA	UT-B

Data were obtained from previously published material or database mining for gene expression, or were generated via RT-PCR (see supplementary material Table S2). For each tissue and each species, PCRs were run on three to four individuals.
*Kakumura et al. (2009); ‡*Bufo marinus* (Konno et al., 2006), *Rana sylvatica*, *Rana pipiens*, *Rana septentrionalis* (Rosendale et al., 2014); §RT-PCR from zebra finch tissues and *in silico* analysis in chicken ENSGALG00000001705; ¶Shayakul et al. (2013).

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Other tissues

Surprisingly, across vertebrates a number of tissues not traditionally associated with any impressive ureogenic capacity (brain, intestine, testis, heart) do also express at least one urea transporter (Table 1). In general, the intestine is not considered to be a major site of urea synthesis (see Bucking et al., 2013a,b); however, as discussed previously, in some animals the guts play an important role in nitrogen salvaging, particularly in herbivorous species, and thus the presence of UTs in this tissue may be important for the transport of urea into the intestinal lumen allowing uptake by microorganisms (Singer, 2003; Stewart et al., 2005). The brain may also seem like an odd tissue to consistently express UTs across species, but given the central importance of arginine and its derived metabolism, it may not be as surprising to find UTs in this tissue (Berger et al., 1998; Sands, 2003; Weisinger, 2001). Indeed, glutamine, glutamate, γ-aminobutyrate as well as polyamines, all derived from arginine, play a predominant role in both neurotransmitter function and cell integrity (Weisinger, 2001). Further, neuronal tissues are fairly delicate and it is not hard to imagine how excessive urea accumulation could impair their function.

The presence of UTs in testis is also interesting, as this is not a tissue usually associated with a functional OUC, but rapid cellular proliferation during spermatogenesis may increase the need for polyamine synthesis and therefore the need for excreting excess urea produced as a by-product (Fenton et al., 2002; Tsukaguchi et al., 1997). In fact, mice deficient in UT-B clearly show an accumulation of urea in the testes, supporting the role of UTs in exporting urea out of this tissue (Guo et al., 2007). In the heart, it has been suggested that urea can have cardioprotective effects in both rat and dogfish, and thus the presence of UTs in the heart under normal conditions may be advantageous (Wang et al., 1999). Furthermore, in mammals, there is an upregulation of cardiac UT expression in pathologies promoting urea production in this tissue, such as an increased polyamine synthesis in the development of cardiac hypertrophy (Duchesne et al., 2001).

Embryonic stages

Through genomic databases and literature searches, it has become apparent that a number of metazoans express UTs early in their development, including birds (chicken, flycatcher), reptiles (chinese turtle, anole), fishes (zebrafish, tilapia and gars) and even mammals (Saunders et al., 2013). Not surprisingly, all these lineages are ureogenic at some point in their early development (Gresham et al., 1971, 1972; Needham et al., 1935; Packard et al., 1977; Wright et al., 1995). However, it is important to note that this capacity to produce urea during embryogenesis is not solely related to ammonia detoxification as previously suggested (Griffith, 1991). It is clear that in several species, such as teleost fish, some of the urea produced by the embryo is derived from ammonia via the OUC (Dépêche et al., 1979; LeMoine and Walsh, 2013). However, the proportion of urea produced by other pathways, particularly in animals that do not express or possess a complete urea cycle at this stage of development, deserves renewed attention. For example, in the chick embryo, most of the urea is produced via argininolysis, as they lack a functional OUC (Needham et al., 1935). Embryogenesis is a period of rapid growth where cells divide and proliferate. As evidenced by a number of studies, polyamine synthesis is not only an essential component of cell growth and division but is also absolutely required during embryogenesis in various taxa (see Heby, 1981, for a review). And as outlined earlier, one of the metabolic pathways leading to polyamine synthesis requires argininolysis and produces urea as a by-product. Furthermore, in a number of vertebrate embryos, regardless of the pathways responsible for urea production, the urea is segregated away from the embryonic body, either the yolk sac or the allantois (Clark, 1953; Fisher and Eakin, 1957; Sartori et al., 2012; Steele et al., 2001), strongly suggesting that urea-transporting mechanisms, potentially mediated by UTs, are at play early on in vertebrate development.

Conclusion and perspectives

The physiological considerations for the role of the UT family in vertebrates have been primarily concerned with the role of urea in concentrating urine in mammals, as well as in whole-animal urea excretion mechanisms in other lineages. However, the availability of molecular data both from a genomics point of view and from an expression standpoint provides us with new datasets that allow us to explore the evolution and function of UTs in vertebrate lineages.

Virtually all living cells have the metabolic machinery to be ureogenic. While a lot of attention has been focused on ureotelic vertebrates and urea production through the OUC, other ureogenic pathways are clearly important for homeostasis at the cellular and tissue level. For example, tissues such as the brain, testes or embryo proper are likely to rely on argininolysis to produce essential components for their function and proliferation, and in all these tissues, there is at least one UT mRNA expressed in most vertebrate species investigated to date. We therefore suggest that besides the association of the UT with typical ureogenic tissues (i.e. the liver via the OUC) and osmoregulatory and excretory tissues, it may be important to consider other ureogenic pathways (i.e. argininolysis) that are essential to tissue function to further understand the physiological role and evolutionary history of the UT in vertebrates. The need to remove urea from these tissues could be due to at least two potential pressures at the molecular level: (1) if urea is allowed to accumulate to levels high enough to lead to protein destabilization, an accumulation of a counteracting stabilizing co-solute (e.g. TMAO) is required; (2) although the arginase reaction equilibrium is especially poised in the direction of urea production, accumulation of urea, a terminal end-product, cannot be allowed to occur to a level that might ‘back up’ these critical synthetic reactions via simple mass-action effects.

In addition to these physiological considerations, a number of important structural and functional questions remain. First, as mentioned earlier, there is little understanding of the multimeric structure of the UT in vertebrates. Although the structure of the UT-A1 (two UT single units in tandem) would suggest that the transporter could function as a dimer, the trimeric and dimeric structures of bacterial homologues could imply that different configurations are possible (Levin et al., 2009; Raunser et al., 2009). Further, the possibility of heterologous multimerization between the different UT isoforms has not been investigated to date. Although some tissues exhibit highly differentiated expression patterns, it is interesting to note that in a variety of species different UTs are co-expressed in a number of tissues. This co-expression could suggest that heterologous multimers of the transporter could indeed be functional and affect the properties and functionality of the multimeric UT.

The above brings us to the second elephant in the room: different UT isoforms may have different kinetics and properties that could generate transport capacities specific to the tissues in which they are expressed. As it is beyond the scope of the current review, we direct the reader to recent work discussing in more detail UT kinetics (MacIver et al., 2008; Stewart, 2011). In brief, it is established that the UT-A and UT-B proteins slightly differ in their susceptibility to phloretin, UT-B being more sensitive to this inhibitor (Martial et al., 1996). It is also suggested that UT-B has asymmetric transport

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capacities, promoting faster efflux of urea from red blood cells than the capacity for intracellular import (Macey and Yousef, 1988). These differences highlight a very important concept in UT characteristics, not only in terms of the overall capacity of these transporters but also in their respective roles and potential physiological function put forward previously. Indeed, in the case where a tissue or cell is better off iso-ureic with the external environment, then a UT-A-type transporter is probably a better choice to allow rapid equilibration of the tissue with plasma content. In contrast, a tissue that is more prone to damage by urea accumulation (e.g. testes, embryonic body) would need to quickly excrete urea rather than equilibrating to extracellular urea concentration and may use an asymmetric type of UT. The reasoning would be similar for tissues that reabsorb urea, for example the rainbow trout kidney, where an asymmetric transporter that facilitates the uptake of urea would allow a reduction in the urinary excretion of urea. In addition, some UTs may be more prone to localize to either the basolateral or apical membranes of epithelial cells, thereby conferring distinct permeabilities to these membranes and allowing urea flux in one or the other direction. While some advances have been made via immunohistochemistry to locate the UT in mammalian kidney (Bagnasco et al., 2001; Nielsen et al., 1996) and the fish gill (Bucking et al., 2013a,b), the cellular orientation of UTs on epithelial cells will continue to be a productive area of research for years to come.

Thus, now that we have examined the large UT repertoire in vertebrates, one can only wonder at the multiple possibilities due to not only the gene registry but also the multiple splice variants and potential for multimerization of these transporters and how these could affect urea handling in these animals. Hopefully, with modern techniques and the use of emerging model organisms, some of these questions can start to be addressed. At this point in time, our understanding of the UT physiological role across vertebrate taxa (excluding mammals) is only in its infancy despite the recent advances and productive efforts made in this field of research.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
C.L.M. and P.J.W. both participated in the conception and design of the experiments, C.L.M. performed the experimental work. C.L.M. and P.J.W. interpreted the results, drafted and revised the manuscript.

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Table S1. Urea transporter sequences used in the phylogenetic analysis.

Species	Class	Isoform	Chr. location	Accession
Desulfovibrio vulgaris	Deltaproteobacteria	aUT	N/A	WP_006042029.1
Acanthamoeba castellanii	Amoeba	aUT	NA	XP_004338489.1
Capsaspora owczarzaki	Filasterea	aUT	NA	XM_004364888.1
Aphanomyces astaci	Oomycota	aUT-1	NA	ETV67386
Saprolegnia parasitica	Oomycota	aUT-2	NA	KDO26855
Emiliania huxleyi	Prymnesiophyceae	aUT-1	NA	XP_005793577.1
Thalassiosira oceanica	Coscinodiscophyceae	aUT	NA	EJK73138
Thalassiosira pseudonana	Coscinodiscophyceae	aUT-1	15	XP_002294089.1
		aUT-2	7	XP_002295777.1
Strongylocentrotus purpuratus	Echinoidea	aUT	N/A	XP_786452
Saccoglossus kowalevskii	Enteropneusta	aUT	N/A	XP_006815841
Branchiostoma floridae	Leptiocardii	aUT	N/A	XP_002606218
Bombus terrestris	Insecta	aUT	N/A	XP_003400996
Bombus impatiens	Insecta	aUT	N/A	XP_003486973
Apis mallifera	Insecta	aUT	N/A	XP_006558393
Megachile rotundata	Insecta	aUT	N/A	XP_003701363
Camponotus floridanus	Insecta	aUT	N/A	EFN65642
Daphnia pulex	Insecta	aUT	N/A	EFX74392
Leucoraja ocellata	Chondrichthyes	UT-A	N/A	AAL12243
Triakis scyllium	Chondrichthyes	UT-A	N/A	BAC75980
Dasyatis sabina	Chondrichthyes	UT-A	N/A	AF443781_1
Chiloscyllium griseum	Chondrichthyes	UT-A	N/A	CDO19228
Squalus acanthias	Chondrichthyes	UT-A	N/A	AF257331_1
Callorhinchus milii	Chondrichthyes	UT-A	N/A	BAH58773
		UT- D1	N/A	BAH58775
		UT-C	N/A	BAH58777
Hydrolagus colliei	Chondrichthyes	UT-A	N/A	AEK49224
		UT- D1	N/A	AEK49225
Opsanus beta	Actinopterygii	UT-A	N/A	AAD53268.2
Porichthys notatus	Actinopterygii	UT-A	N/A	AGA93882.1
Xiphophorus maculatus	Actinopterygii	UT-A	NA	ENSXMAP00000007361
		UT-C	N/A	ENSXMAP00000008889
		UT-D2	N/A	ENSXMAP00000005111
Oryzias latipes	Actinopterygii	UT-A	9	ENSORLP00000011043
		UT-C	18	ENSORLP00000005116
		UT-D2	12	ENSORLP00000006253
Takifugu rubripes	Actinopterygii	UT-A	NA	ENSTRUP00000026150
		UT-C	NA	ENSTRUP00000019396
Tetraodon nigroviridis	Actinopterygii	UT-A	12	ENSTNIP00000018473
		UT-C	NA	ENSTNIP00000003172
Gasterosteus aculeatus	Actinopterygii	UT-A	XIII	ENSGACP00000012975
		UT-C	VII	ENSGACP00000026274
		UT-D2	XIV	ENSGACP00000021320
Gadus morhua	Actinopterygii	UT-A	N/A	ENSGMOP00000014981
Anguilla japonica	Actinopterygii	UT-A	N/A	BAC53976
		UT-C	N/A	BAD66672

Danio rerio	Actinopterygii	UT-A	5	ENSDARP00000068116
		UT-C	7	XP_001921059
Oreochromis niloticus	Actinopterygii	UT-A	N/A	ENSONIT00000017261
		UT-C	N/A	ENSONIP00000011541
Lepisosteus oculatus	Actinopterygii	UT-A	LG2	ENSLOCT00000007597
Poecilia formosa	Actinopterygii	UT-A	N/A	ENSPFOP00000017441
		UT-D2	N/A	ENSPFOP00000014299
		UT-C	N/A	ENSPFOP00000003520
Latimeria chalumnae	Sarcopterygii	UT-A	N/A	XP_006007026
Protopterus annectens	Sarcopterygii	UT-A	N/A	ACH85194
Rana pipiens	Amphibia	UT-A	N/A	AFE48182
Rana septentrionalis	Amphibia	UT-A	N/A	AFE48183
Rana sylvatica	Amphibia	UT-A	N/A	AFE48181
Rana esculenta	Amphibia	UT-A	N/A	CAA73322
Rhinella marina	Amphibia	UT-A	N/A	BAE16706
Xenopus Tropicalis	Amphibia	UT-A	N/A	ENSXETP00000059453
Taeniopygia guttata	Sauropsida	UT-A	Z	ENSTGUP00000001662
Gallus gallus	Sauropsida	UT-A	Z	ENSGALP00000002617
Pelodiscus sinensis	Sauropsida	UT-A	N/A	ENSPSIP00000013054
Alligator sinensis	Reptilia	UT-A	N/A	XP_006034341
Homo sapiens	Mammalia	UT-A	18	ENSP00000255226
		UT-B	18	ENSP00000390637
Canis familiaris	Mammalia	UT-A	7	ENSCAFP00000026068
		UT-B	7	ENSCAFP00000033649
Bos taurus	Mammalia	UT-A	24	ENSBTAP00000002712
		UT-B	24	ENSBTAP00000044109
Mus musculus	Mammalia	UT-A	18	ENSMUSP00000025434
		UT-B	18	ENSMUSP00000125114

Table S2. Species-specific primers used in RT-PCR for UT tissue distribution.

Species	Isoform (accession)	Forward Primer 5'-3'	Reverse Primer 5'-3'
Taeniopygia guttata	UT-A (ENSTGUP00000001662)	ctacctggctgcttcaggac	ggacagggagctgttgtagc
Danio rerio	UT-A (ENSDARP00000068116)	agatggtgggcactcaatgg	ggagaccaaccaaaatgccg
	UT-C (XP_001921059)	aaccctacccactgcaactg	gctgtgaaggtagaggccaa
Opsanus beta	UT-A (AAD53268.2)	cctcaacggctttgtaggaa	gtaccaatttcctgcgttgg
	UT-C (KP122949)	ctctgtactggcccggataa	accccacaggcataaatctg
	UT-D2 (KP122950)	gcttttcctgccaaatgtgt	aatagggatggttggctcct
Squalus acanthias	UT-A (AF257331_1)	caggattatccctggcttca	aagcgcagaaaattgagcat

Flash-frozen tissues obtained from dogfish, zebrafish, zebra finch and toadfish were pulverised in liquid nitrogen and total RNA was extracted, reverse transcribed and subjected to PCR using species-specific primers; the products were run on agarose gels to determine the presence or absence of UT in individual tissues. For each tissue and each species, PCRs were run on three to four individuals.