Introduction

When a particular gene is constrained to a specific function, the appearance of biological novelty demands genetic redundancy. Duplication of pre-existing genes may lead to the establishment of lineage-specific traits and to the development of novel biological functions [1,2,3,4,5]. However, the probability of widening biological functions (neofunctionalisation) is expectedly lower than the chance of inactivation (pseudogenisation) [6,7,8] as most amino-acid replacements are more likely neutral or deleterious, rather than leading to any particular adaptive change. Although the majority of gene duplicates result in pseudogenes, many remain functionally active longer than it would be expected by chance. This observation led to the development of the subfunctionalisation model [9,10], according to which the accumulation of complementary loss-of-function mutations within regulatory segments of both members would facilitate their preservation while maintaining the original function. In case of preserving the parental function, duplicates may act as backup compensation copies to buffer against the loss of a functionally related gene [11,12].

The current availability of several genome sequences allows the study of the evolutionary steps underlying the expansion of a gene family by detailed characterisation of lineage-specific expansions. We focused our attention on the mammalian metallothionein family for a number of reasons. MTs are metal-binding proteins involved in homeostasis and the transport of essential metals, more specifically, in protecting cells against heavy metals toxicity [13,14], having thus a critical role in many biological processes. In mammals, four tandemly clustered genes (MT1 to MT4) are present and are known. Although all genes encode for conserved peptide chains that retain 20 invariant metal-binding cysteines, MT3 and MT4 seem to have developed additional properties relatively to MT1 and MT2, such as protection against brain injuries [15,16] and epithelial differentiation [17], respectively. Finally, during the evolution of the lineage that led to modern humans, MT1 has undergone further duplication events that have resulted in 13 younger duplicate isoforms [18]. The co-existence of younger and older duplicates is reconstructed by sequence homology with a functional duplicate revealing that loss of invariant cysteines is the most frequent event accounting for pseudogeneisation. Expression analyses based on EST counts and RT-PCR experiments show that, as for MT1 and MT2, human MT3 is also ubiquitously expressed while MT4 transcripts are present in brain, testes, esophagus and mainly in thymus. Polymorphic variation reveals two deleterious mutations (Cys30Tyr and Arg31Trp) in MT4 with frequencies reaching about 30% in African and Asian populations suggesting the gene is inactive in some individuals and physiological compensation for its loss must arise from a functional equivalent. Altogether our findings provide novel data on the evolution and diversification of MT gene duplicates, a valuable resource for understanding the vast set of biological processes in which these proteins are involved.

Abstract

Metallothioneins (MT) are small proteins involved in heavy metal detoxification and protection against oxidative stress and cancer. The mammalian MT family originated through a series of duplication events which generated four major genes (MT1 to MT4). MT1 and MT2 encode for ubiquitous proteins, while MT3 and MT4 evolved to accomplish specific roles in brain and epithelium, respectively. Herein, phylogenetic, transcriptional and polymorphic analyses are carried out to expose gains, losses and diversification of functions that characterize the evolutionary history of the MT family. The phylogenetic analyses show that all four major genes originated through a single duplication event prior to the radiation of mammals. Further expansion of the MT1 gene has occurred in the primate lineage reaching in humans a total of 13 paralogs, five of which are pseudogenes. In humans, the reading frame of all five MT1 pseudogenes is reconstructed by sequence homology with a functional duplicate revealing that loss of invariant cysteines is the most frequent event accounting for pseudogeneisation. Expression analyses based on EST counts and RT-PCR experiments show that, as for MT1 and MT2, human MT3 is also ubiquitously expressed while MT4 transcripts are present in brain, testes, esophagus and mainly in thymus. Polymorphic variation reveals two deleterious mutations (Cys30Tyr and Arg31Trp) in MT4 with frequencies reaching about 30% in African and Asian populations suggesting the gene is inactive in some individuals and physiological compensation for its loss must arise from a functional equivalent. Altogether our findings provide novel data on the evolution and diversification of MT gene duplicates, a valuable resource for understanding the vast set of biological processes in which these proteins are involved.

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transcripts were: 5

heat-maps were constructed with an in-house tool using average
MT2
PCR primers used for amplification of the
TCTGCCTCAGCTGCCTCT3
GAATGTGTCTGCATGT3
62
GGCACAGCCCGG3

RT-PCR and expressed sequence tag (EST) analyses
Total RNA from 15 human tissues was obtained from Ambion
(FirstChoice Human Total RNA Survey Panel). The complemen-
tary DNA (cDNA) was synthesized with random hexamer primers
from 2 µg of total human RNA using the RETROscrip First Strand
Synthesis Kit (Ambion) according to the manufacturer’s instructions.
PCR primers used for amplification of the MT family and flanking
neighbours (BBS2 and NUP93) in humans and mice was
performed using NCBI (Homo sapiens build 36.2 and Mus musculus
build 37.1) [26] and Ensembl (release 56) genomic coordinates.

Results
Evolutionary history of the MT cluster in mammals
The phylogeny of the MT family in mammals was reconstruc-
ted with Bayesian and maximum likelihood methods both
approaches resulted in a similar topology (Fig. 1 and Figure S1)
revealing the robustness of the inference. Tree topology, supported
by high values of posterior probabilities (Fig. 1), point clearly
to two rounds of duplication occurring at the MT family creating
MT4 first and the ancestor of MT1/MT2/MT3. In a second
round of duplication, MT3 diverged from the MT1/MT2
ancestor. These data are in agreement with previous observations
[31] which concluded that MT duplication occurred before
mammalian radiation. However, because MT genes from birds
and reptiles group within the MT1/MT2/MT3 cluster, it is
possible to consider a more ancient origin for MT4, and therefore,
its loss in non-mammalian land vertebrates. An alternative
explanation is that MT4 is thus a mammalian-specific gene which
has been accumulating a pronounced number of replacements
assigning its sequence to a basal position in both Bayesian and ML
phylogenies.
Extant mammalian genomes seem to carry a single copy of
MT2, MT3 and MT4, while several MT1 copies are found in some
species. The highest number of MT1 genes was found in the
genome of primates indicating they have arisen in recent
duplication events. The detailed genomic organization of the
MT cluster in humans and mice provides a good example (Fig. 2).
In both species, genes are oriented as cent-
MT4-MT3-MT2-MT1
tel and flanked by
BBS2 and NUP93, revealing an evolutionarily
conserved arrangement of the cluster. Still, a marked difference
distinguishes both genomes. While mt1 did not expand in mice,
humans carry 15 arrayed duplicates (MT1A to MT1J), MT1L,
MT1M and MT1X), five of which (MT1L, MT1J, MT1D, MT1C
and MT1H) have been predicted to be no longer active forms.
Overall, this corresponds to a genomic expansion of about 66 Kb
in humans.
Characterisation and divergence of mammalian MT
proteins
Mammalian MTs are proteins with 20 invariant cysteines
(Fig. 3A) which are responsible for the binding and sequestrat-
ion of zinc (Zn), cadmium (Cd) and copper (Cu), among other metals.
A total of 9 and 11 cysteines are required to form protein-domains
that bind three and four ions (Fig. 3B). As documented previously
[32,33] and illustrated here (Fig. 3B), these residues are involved in
metal binding through their thiol (-SH) moieties, which must be
oriented towards the inside of the metal clusters whenever the ions
are sequestered [34]. In humans and mice, MT1 and MT2 are 61-
residue proteins (Fig. 3A). The MT3 holds an extra residue in the
β domain (Thr5), which is important for its neuroinhibitory
activity [35,36], and a six-residue long insertion in the α domain, whereas MT4 shares an additional residue in the β domain (Glu5) (Fig. 3A).

In order to obtain a clear picture of the amino acid conservation between all pairs of active MTs, identity scores were calculated in human/mouse comparisons (Fig. 4). As shown, MT2, MT3 and MT4 orthologues share 86%, 87% and 94% of residue identity, respectively. Among human MT1 duplicates, MT1E showed the highest identity (85%) with the mouse M1. Protein identity scores for MT3 resembles that observed in MT1 and MT2 while MT4 orthologues are strongly conserved in their aminoacid sequences (94% of residue identity between human and mouse). Previously, it was suggested that the preservation of MT4 sequence (only 4 out of 62 residues differed between human and mouse proteins) results from functional constraints [37] involved in epithelial cell differentiation [17]. Phylogenetic analyses (Fig. 1 and Figure S1) show that MT4 resulted from an old event of duplication and the high degree of sequence conservation between human and mouse orthologues strongly points to a role which seems to have been functionally important in mammalian evolution.

About the pseudogenisation incidents

An important aspect related to the fate of a gene copy is the identification of the type of replacements leading to the pseudogenisation of functionally active genes. From its genomic sequence, we would be able to reconstruct the ancestral functional open reading frame and, at the same time, discern the panel of mutational events that have accumulated over time. To reconstruct the open reading frame of all the five MT1 pseudogenes, the corresponding genomic sequences were aligned with each of the MT1E exons separately, followed by manual inspection of sequence homology (Fig. 5). Because any attempt to disclose the impact of nonsynonymous replacements is not straightforward unless complemented with additional functional assays, we focused our attention on obvious damaging mutations, such as (a) replacement of invariant cysteine residues, (b) introduction of aromatic residues, as well as (c) premature stop codons, indels and mutations at the consensus donor (GT) or acceptor (AG) splice sites. Because these are shared features among all functional genes, our inferences are not constrained by the functional template that could have been chosen to infer the reading frame of each pseudogene.

We detected a total of 12 deleterious mutations within the predicted reading frame of MT1 pseudogenes, eight of which would result in the cysteine replacement and, in some cases, the inclusion of a premature stop codon (Cys5Tyr, Cys15Tyr, Cys24Tyr, Cys26X, Cys37Tyr, Cys41X, Cys50X, and Cys60Arg) covering MT1JP, MT1CP, MT1DP and MT1LP, three non-

Figure 1. Bayesian phylogenetic analysis of the MT family. The gene tree was constructed using coding sequences from the Ensembl database (Table S1). MT1/MT2, MT3 and MT4 clusters are represented in blue, red and green, respectively. Posterior probability values are given for branch support. Scale bar stands for the number of replacements per site. doi:10.1371/journal.pone.0018487.g001
cysteine codons that would encode for an aromatic residue (Gly11Phe, Ser18Phe and Ser35Phe) in MT1IP and MT1CP, and a 1-bp deletion at the C-terminal domain in MT1IP. Since cysteine replacement either with tyrosine or with a stop codon involves only a single nucleotide substitution, these residues are expected to often contribute to MT1 pseudogeneization.

Outside the coding sequence, two mutations were found at the donor and acceptor splice site of MT1CP and MT1IP, respectively. Regarding the number of mutations, MT1CP is the pseudogene harbouring the highest number of events (six in total), followed by MT1IP and MT1DP (with three mutations each), MT1JP (two mutations) and finally by MT1IP, which would encode a truncated protein due to a premature stop codon.

Expression profile of the MT genes in humans and mice

Since gene duplication often results in a diversified spatiotemporal pattern of expression of duplicate family members [38,39,40,41,42] we next examined the MT transcription pattern using EST data for 30 human and mouse tissues as a metric of basal expression for all functional genes. Previous data have shown that MT1 and MT2 are ubiquitously expressed in both species whereas MT3 and MT4 present a confined expression in human and mouse brain [43] and in mouse epithelial tissue [17] respectively, although data regarding expression of MT4 in human tissues is still missing in the literature. The EST records were assembled in a diagram (Fig. 6A) that, in general terms, strongly overlaps the literature data. For instance, mouse mt1 and mt2 seem to be as widely expressed as the human MT2, a gene that has been proposed to have a housekeeping role for heavy metal homeostasis in every cell [44]. In humans, the expression pattern of MT2 clusters with MT1E and MT1X, the two duplicates that reveal the most wide pattern of basal expression [45]. The remaining genes revealed a more confined pattern of basal expression. For instance, constitutive expression of MT1B seems to be restricted to connective and blood tissues, whereas MT1A is highly expressed in the intestine and adipose tissue and less in uterus, eye, liver and lung. This analysis also rank MT1H, MT1F and MT1G in an intermediate position, showing a pattern that is not as wide as that of MT1E and MT1X but not so restricted as that of MTB, MT1A and MT1M either. Although the lack of ESTs in particular tissues may indicate difficulties to distinguish between different MT1 transcripts or bias in tissue representation, these caveats do not necessarily challenge the tissue-specificity generally observed in most MT1 duplicates, possibly playing distinct roles in distinct cell types as was suggested before [44,46].

In contrast to MT1 and MT2, both MT3 and MT4 are constitutive tissue-specific isoforms that do not respond to metal-induction [17,44,47,48,49]. The EST data (Fig. 6) although supporting high expression of MT3 in human and mouse brain tissues also reveal abundant expression in other tissues as well. Concerning the MT4 profile, EST records agree with previous findings that documented the stratified squamous epithelium of digestive and reproductive systems as the main source of gene transcripts in mouse [17]. Thus far, no data exist on the expression of MT4 in humans (reviewed in [31]) and the EST surveillance has not detected expression in any tissue. These intriguing observations directed a more refined analysis concerning MT4 expression in humans that was here accomplished by RT-PCR in 15 human tissues. For comparative purposes we also assayed the expression of MT2 and MT3 in the same tissue collection (Fig. 6B). As expected, transcripts related with MT2 were observed in all tissues analysed. A similar scenario was observed for MT3 which is here demonstrated to be as ubiquitously expressed as is MT2. On the other hand, the detection of MT4 transcripts was only possible in four tissues (brain, testes, thymus and esophagus), with an evident higher expression in thymus, whose medullar component is mainly composed of epithelial cells [50]. Although the RT-PCR itself cannot provide exact quantitative inferences, it is possible to infer...
that in all the tissues where MT4 transcripts were detected, the expression level resulted lower than that of MT2 and MT3 with the exception of thymus.

Human polymorphic variability at the MT cluster

Although it is well established that functional MTs preserve a sequence with 20 invariants cysteines, no studies have thus far established the polymorphic status of the remaining residues. To achieve such information, we retrieved the available information on all human active proteins regarding nonsynonymous replacements (Table 1). Several nonsynonymous variants were found in MT1 duplicates and in MT2, but none is documented for MT3. Although a link between any of these replacements and a functional perturbation is not straightforward without comple-

![Figure 3. Sequence comparison and structural features of MT proteins.](image)

![Figure 4. The amino acid identity matrix for human and mouse proteins.](image)
mentary experimental assays, it should be mentioned that, in most of the cases the conserved cysteine residues remain unchanged and the replacement does not introduce an aromatic amino acid. The exceptions were observed in MT1B and MT4. In the MT1B case, a putative deleterious Cys19Ser (rs61744104) is indicated as polymorphic in humans although no additional data concerning the allelic frequencies are available in the databases. In the MT4 case, two candidate deleterious mutations were detected, Cys30Tyr (rs666636) and Arg31Trp (rs666647), both of which result in the introduction of aromatic amino acids along with the substitution of a critical cysteine at position 30. Taking into account the functional requisites of MTs, any of these mutations would ultimately result in an impaired metal-binding protein. In contrast with the MT1B case, allelic frequencies for these two polymorphisms are available and were retrieved from the HapMap (Table 2). Allelic frequencies of Cys30Tyr (rs666636) and Arg31Trp (rs666647) in 11 populations are presented in Table 2. In European populations up to 8.5% of the individuals carry a putatively deleterious allele. Frequencies are even higher in African and Asian populations (up to 30%). Genotype evaluation in all the populations showed that every chromosome that contains the Trp31 allele also contains the Tyr30, but not vice versa, which points to Cys30Tyr as the oldest variant and Arg31Trp as appearing afterwards in the same background.

**Discussion**

The history and fate of post-duplication events in the mammalian evolution was herein explored by the study of MT family members. After a duplication event, newly arisen genes can follow distinct evolutionary paths: if the parental gene is maintained active, redundant duplicates can escape purifying selection and start to accumulate loss-of-function mutations resulting in pseudogeneisation; less frequently, particular replacements may direct new genes into novel functions. Subfunctionalisation can also occur if parental and duplicates retain function but become distinct and complementary in their spatiotemporal pattern of expression.

Mammalian MT1 and MT2 are conserved proteins that play a critical role in heavy-metal homeostasis and are transcriptionally induced by metal [51] and glucocorticoids [52]. While most of the mammals show a single MT1 and MT2 copy that evolved through a duplication event, primates harbor multiple MT1 copies (Fig. 1). In humans, MT1 expansion resulted in a total of 13 tandemly arranged genes, five of which are known or predicted to be pseudogenes, while the remaining eight are still functionally active (Fig. 1). Since its discovery [54], MT3 has been frequently associated with the protection against neuronal injury [15,16]. The mammalian MT3 protein shows a characteristic insertion of six residues at the α-domain when compared to that of MT1 and MT2 (Fig. 3) and an extra residue in the β domain (Thr), which is responsible for neuron growth inhibitory activity in Alzheimer disease [35,36]. Although the expression of MT3 has been almost exclusively related to brain tissues, we demonstrate that MT3 is a ubiquitously expressed gene. These results would drive future investigations on the involvement of MT3 in other cellular processes. In this regard, it is worth mentioning that Mt3 associates with other proteins in mouse brains as part of a multiprotein complex [55] suggesting function diversification and involvement in various physiological processes.

The most recently discovered family member, MT4, retains a high degree of conservation between humans and mice (Fig. 4), yet it shows the highest sequence divergence when compared with any
However, the detection of structurally disrupting mutations at polymorphic proportions (Table 1) predicts that MT4 is inactive in some individuals. If that is the case, might the role of MT4 be performed by another family member? To address this possibility, the metal binding properties of MT4 were explored in the literature data. It has been shown that mouse Mt4 retains the capacity to bind Zn [17,37], Cd and Cu as the ubiquitously expressed Mt1/Mt2, although the affinity to Cu is higher [37,56]. Furthermore, it showed similar characteristic metal-thiolate clusters and solvent accessibility as Mt1 [57]. Extrapolating these properties to the human protein, for which no data of such detail are available, it is tempting to assume that the loss (pseudogeneization) of MT4 can be compensated by functional equivalents. In this context, MT1 and MT2 would be the most likely candidates for a number of reasons. First, the metal binding properties of Mt1 and Mt2 overlap that of Mt4 in mice [57]. Second, it has been demonstrated that some MT1 duplicates have cellular specificity [44,46] and some of them are expressed in epithelium. Third, previous experiments in Drosophila melanogaster demonstrated that the number of functional gene duplications correlates to the resistance to Cd [13,58] and Cu [13] as a direct consequence of the increased gene expression. Taken these data together, is thus possible that additional MT1 duplicates may have assumed the specialized role of the inactivated protein in humans. In such a case, it is likely that some MT1 genes may act as compensatory backup copies that replace MT4 in individuals.
carrying deleterious mutations. Accordingly, it is thus further possible to infer that the compensatory mechanism implies the regulation of gene expression as observed in *D. melanogaster*.

Table 2. Hapmap allelic frequencies of MT4 Tyr30 (rs666636)
and Trp31 (rs666647) in human population.

| Population | TYR<sup>30</sup>-A allele | TRP<sup>31</sup>-T allele |
|------------|---------------------------|--------------------------|
| African    |                           |                          |
| ASW        | 0.189                     | 0.189                    |
| LWK        | 0.303                     | 0.244                    |
| MKK        | 0.155                     | 0.150                    |
| YRI        | 0.161                     | 0.124                    |
| Asian      |                           |                          |
| CHB        | 0.298                     | 0.298                    |
| CHD        | 0.288                     | 0.282                    |
| JPT        | 0.267                     | 0.265                    |
| GHI        | 0.045                     | 0.045                    |
| European   |                           |                          |
| CEU        | 0.085                     | 0.058                    |
| TSI        | 0.034                     | 0.034                    |
| American   |                           |                          |
| MEX        | 0.05                      | 0.05                     |

Population description as indicated in Hapmap: ASW, African ancestry in Southwest USA; CEU, Utah residents with Northern and Western European ancestry from the CEPH collection; CHB, Han Chinese in Beijing, China; CHD, Chinese in Metropolitan Denver, Colorado; GHI, Gujarati Indians in Houston, Texas; JPT, Japanese in Tokyo, Japan; LWK, Luhyia in Webuye, Kenya; MEX, Mexican ancestry in Los Angeles, California; MKK, Maasai in Kinyawa, Kenya; TSI, Toscans in Italy; and YRI, Yoruba in Ibadan, Nigeria.

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Tracing the evolutionary history of a gene after duplication often leaves more questions than answers. Some of those answers are easily obtained by direct read of DNA or protein sequences while some other depend on additional information which was here gathered for the *MT* family. Data on phylogeny, expression and intra-specific polymorphic information are necessary to drive hypotheses regarding the gain, loss and change of function of duplicated genes. The application of such a network of information, as is the case of this study, is thus necessary to extend the knowledge about the evolutionary fate of genes originated by duplication events.

Supporting Information

Figure S1 Maximum likelihood analysis of the *MT* family. The gene tree was constructed using coding sequences from the Ensembl database (Table S1). *MT1A/MT2, MT3* and *MT4* clusters are represented in blue, red and green, respectively. Branch support was estimated by bootstrap. Scale bar: number of replacements per site.

Table S1 Metallothionein gene transcripts annotated in the Ensembl database (release 36, Sep 2009) as human orthologues of *MT1E, MT1M, MT1A, MT1B, MT1F, MT1G, MT1H, MT2, MT3* and *MT4* in mammals. *MT* sequences from fishes, birds and reptiles are shown at the bottom of the table.

Author Contributions

Conceived and designed the experiments: AA LA. Performed the experiments: AM JC RM LA. Analyzed the data: AM JC RM RMS AA. Contributed reagents/materials/analysis tools: RM AA LA. Wrote the paper: AA LA.
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