

# Human pentatricopeptide proteins

## Only a few and what do they do?

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**Abbreviations:** PPR, pentatricopeptide; LRPPRC, leucine-rich PPR cassette; POLRMT, mitochondrial RNA polymerase; MRP, mitochondrial ribosomal protein; PTCD, pentatricopeptide domain containing; MRPP3, mitochondrial RNase P protein 3

Pentatricopeptide repeat (PPR) proteins constitute a large family of RNA-binding proteins that contain a canonical 35 residue repeat motif. Originally identified in *Arabidopsis thaliana*, family members are found in protists, fungi and metazoan but are by far most abundant in plant organelles. Seven examples have been identified in human mitochondria and roles have been tentatively ascribed to each. In this review, we briefly outline each of these PPR proteins and discuss the role each is believed to play in facilitating mitochondrial gene expression.

### Introduction

Pentatricopeptide repeat (PPR) proteins were first identified when Peeters and Small were searching the *Arabidopsis thaliana* genome for proteins that might be targeted to mitochondria or chloroplasts. To their surprise, they uncovered a vast family of proteins with almost 200 members.<sup>1,2</sup> Further analysis has shown the PPR protein family to be one of the largest known, with two subfamilies and four subclasses containing between 400–600 independent proteins localized to angiosperm organelles.<sup>3</sup> Since that time, PPR proteins have been identified in many other organisms but their numbers are often limited to approximately 10 per organism. Plants, therefore, appear to be unique in this remarkable expansion of a particular protein family.<sup>3</sup>

**What are PPR proteins?** These proteins are characterized bioinformatically by a canonical motif, a 35 amino acid stretch<sup>1</sup> that can be repeated up to 30 times. Their structural characterization by contrast was hampered for many years, as PPR proteins were notoriously difficult to acquire in a soluble form.<sup>4</sup> More recent efforts, however, have revealed that the PPR protein RF1A from rice conforms to the prediction of an array of  $\alpha$ -helices<sup>5</sup> as does the recently crystallized *Arabidopsis* PRORP1 protein.<sup>6</sup> The other highly conserved feature is the RNA-binding properties that these proteins display, which have clear sequence specificity.<sup>3,7</sup> The latter is critically linked to function as these proteins

are involved in a number of aspects of post-transcriptional RNA processing in organelles, reviewed in reference 8.

**How are PPR proteins distributed across organisms?** Since these proteins are involved in processing of organellar RNA, one facile explanation would be that plants have both chloroplasts and mitochondria compared with humans having only mitochondria. That would inaccurately assume that chloroplasts have the lion's share of the PPR proteins and it would also not account for the 50-fold difference in number of PPR proteins found in plants over humans and other organisms. More appropriate theories, however, have been put forward to try and explain this difference and the rapid expansion of this family in plants.<sup>3,9</sup> One such hypothesis has its origins in nucleocytoplasmic conflict, whereby the organellar genome evolves to gain advantage over its host and the host nucleus co-evolves to restore this balance.<sup>10,11</sup> In many cases, these nuclear “restorer” genes encode PPR proteins.<sup>12</sup> Plants generally have much larger organellar genomes than human mitochondrial DNA, 390–2900 kb in size compared with the human 16.5 kb.<sup>13,14</sup> These are very plastic genomes that can actively recombine, accept foreign DNA by horizontal gene transfer, form multiple circular genomes, expand extensive introns and transcriptomes harbor RNA editing sites, all of which give a wide scope for evolutionary change.<sup>13,15</sup> Unlike the extensive organellar genomes of plants, human mitochondrial DNA (mtDNA) is a small compact genome exhibiting vanishingly low levels of recombination.<sup>14,16,17</sup> Transcription yields polycistronic transcripts that need to be cleaved into the separate tRNAs, rRNAs, and mRNAs but there are few nucleotides between these coding units and there are no spliced intronic sequences. The consequence is that very little non-coding RNA needs to be removed, and there is no RNA that requires editing. Thus, many of the mechanisms that are fundamental to post-transcriptional processing in plants, and possibly the PPR proteins responsible for mediating these, are absent in human mitochondria. However, PPR proteins have also been shown to be involved in transcript stability and to act as translational activators.<sup>7</sup> It is, therefore, possible that we may yet uncover further human PPRs that assist in stabilizing RNA or stimulating its translation.

**So how many PPR proteins are there in man?** In 2008 the list numbered only six human PPR proteins<sup>18</sup> and yeast had even

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fewer with only three identified in *Saccharomyces cerevisiae*.<sup>19</sup> Since then, efforts have been made to improve the predictive algorithms in an attempt to uncover PPR proteins that have hitherto escaped identification (reviewed in ref. 4). This task has been made harder as a computational comparison of PPRs from different organisms has shown that predictive methods for one organism are suboptimal for distinguishing PPRs from other organisms.<sup>19</sup> However, a newly derived iterative hidden Markov model has increased the number of potential yeast PPR proteins from three to 12, all of which are mitochondrial.<sup>19</sup> Other models have also been established that allow prediction of the specific RNA target to which particular PPR proteins bind.<sup>9,20</sup> It is hoped that such predictive bioinformatic algorithms may improve our understanding of PPR function and specificity. In time, this may have a therapeutic use, as PPRs could be genetically manipulated to alter their specificity in order to target and neutralize aberrant RNAs.<sup>9</sup>

Despite these efforts, the number of human PPR proteins remains low at only 7 (Table 1), all are found localized to mitochondria and either by prediction or experimental evidence all are RNA binding proteins. Although the number of identified PPR proteins has not increased dramatically, we have greatly increased our detailed understanding of their roles in the mitochondrion and their interaction partners.

### LRPPRC

The first human PPR protein to be identified was the leucine-rich PPR cassette (LRPPRC, also called LRP130) protein, which was identified through an integrative genomics approach demonstrating how combining proteomic/genomic data and RNA expression patterns could inform disease-gene identification.<sup>21</sup> This was swiftly followed by a report linking mutations in LRPPRC with a form of cytochrome *c* oxidase (COX) deficiency (French Canadian Leigh syndrome). In LRPPRC-deficient patient cell lines, Robinson and colleagues observed lowered steady-state levels of mitochondrial transcripts coding for members of complex IV of the respiratory chain.<sup>22</sup> Further work has established that although French Canadian Leigh syndrome affects complex IV, its clinical presentation is distinct from other forms of COX deficiency.<sup>23</sup> Modeling this syndrome by depletion of LRPPRC using shRNA in human fibroblasts also showed lowered levels of almost all mtDNA encoded transcripts with relative sparing of the mitochondrial (mt)-rRNAs.<sup>24</sup> In related work, mice expressing a homozygous C-terminal deleted LRPPRC variant were shown to be embryonic lethal. It was possible to derive embryonic fibroblasts from this model and these too showed decreased COX activity.<sup>25</sup>

LRPPRC expression has also been examined in various tumor samples. Analysis using immunohistochemistry has revealed much higher levels of this protein in the tumor compared with the surrounding normal tissue, together with reduced apoptosis.<sup>26</sup> This anti-apoptotic activity has been observed by other groups,<sup>27</sup> suggesting that LRPPRC may play a role in tumorigenesis.

**How conserved is LRPPRC and how does it work?**  
Bioinformatic analysis of the primary sequence of genes encoding

**Table 1.** Human PPR proteins and reported interactors

PPR protein	PPR domains <sup>30</sup>	Known associated proteins*
LRPPRC	22	SLIRP, ATAD3, C4orf14, ICT1, POLRMT, PGC-1 $\alpha$ , eIF4E, neurofibromin
POLRMT	2	LRPPRC, TEFM, MRPL12, h-mtTFB1.
MRPS27	6	MRPs
PTCD1	8	ELAC2, ICT1
PTCD2	5	
PTCD3	15	ICT1, mtRRF, TEFM, mtlF3
MRPP3	3	mitochondrial RNase P complex

\*Lists only associated proteins described in the main text, not fully comprehensive of all published literature.

LRPPRC indicates that orthologs are restricted to the metazoa, and that the corresponding proteins carry targeting signals that will localize them to mitochondria, although a small fraction has been reported to be localized to the nucleus.<sup>28,29</sup> LRPPRC shows selectivity in nucleotide binding with no detectable affinity for poly(A) and preferential binding for polypyrimidines. This RNA binding is facilitated by the C terminus as was demonstrated by in vitro RNA-binding assays using numerous recombinant truncated LRPPRC variants that differed in their numbers of PPR motifs.<sup>29</sup> This confirmed the importance of the C terminus for function, as had been previously observed in mice,<sup>25</sup> and revealed that only two of the 11 predicted PPR motifs are involved in RNA binding.<sup>29</sup> Prediction of these motifs is not trivial and the estimate has now increased from 16 PPRs for human LRPPRC<sup>28</sup> to 22.<sup>30</sup>

Despite the inability to bind poly(A), LRPPRC appears to be necessary for polyadenylation of mt-transcripts.<sup>31</sup> Depletion of LRPPRC in HeLa cells resulted in reduced polyadenylation and also reduced steady-state levels of virtually all mt-mRNAs.<sup>32,33</sup> This result was recapitulated in a heart-specific disruption of *Lrpprc* in mice that caused mitochondrial cardiomyopathy accompanied by a dramatic decrease in the steady-state levels of mt-mRNAs, only. This decrease reflected a loss of stability and also reduced polyadenylation of the mt-transcripts. The consequence was aberrant mitochondrial protein synthesis, with marked variability in the translatability of different transcripts—increased, decreased or even absent.<sup>31</sup> Loss of poly(A) tails and decreased transcript stability were also observed in *Drosophila* after knockdown of the Bicoid Stability Factor, a PPR protein with homology to LRPPRC.<sup>34</sup> In concert, these data confirm the importance of LRPPRC in the maintenance of mitochondrial gene expression.

In both human and mice, the RNA binding by LRPPRC appears to function as part of a complex together with a much smaller protein, SLIRP.<sup>31,32,35</sup> SLIRP was originally identified as a stem-loop RNA-binding protein that is active in the nucleus, although in fact the majority of the protein partitions to mitochondria,<sup>36</sup> where together with LRPPRC they sequester and stabilize an extra-ribosomal pool of mt-mRNAs protecting them from exonucleolytic degradation prior to translation.<sup>31,35</sup>

In addition to SLIRP, LRPPRC appears to interact with a number of proteins involved in mitochondrial biogenesis,<sup>37</sup>

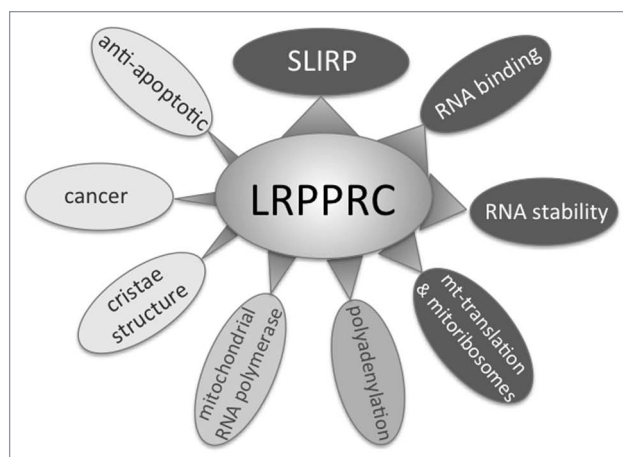
transcript synthesis<sup>38</sup> and also translation as it has been found to immunoprecipitate with a member of the large mitoribosome, ICT1.<sup>39</sup> Consistent with a role in gene expression, SILAC analyses have also identified LRPPRC in complexes with ATAD3, known to be associated with nucleoids, and C4orf14, which is involved in biogenesis of the small subunit of the mitoribosome.<sup>40,41</sup> In contrast to its mitochondrial function, it has been reported to bind the cytosolic proteins translation initiation factor 4E<sup>42</sup> and neurofibromin.<sup>43</sup>

Studies on the overexpression of LRPPRC examined the consequences on mitochondrial biogenesis at the ultrastructural level. Electron microscopy revealed that there was no change in the mitochondrial volume compared with controls but there was a clear remodeling resulting in increased cristae density.<sup>38</sup>

This shows that LRPPRC is involved in a number of different pathways and interacts with numerous proteins, only some of which are related here (Fig. 1). There are a significant number of other reports relating to LRPPRC, including those suggesting it can affect fatty acid oxidation,<sup>38</sup> and interact with mitochondrial RNA polymerase to activate mt-transcription.<sup>33,38</sup> Not all the data concerning LRPPRC are consistent and there are probably more interactions yet to be uncovered, but it certainly appears to be an important mitochondrial protein that plays a role in mt-RNA metabolism.

### POLRMT

The mitochondrial RNA polymerase (POLRMT) is another of the human PPR proteins. It too has a key role in mitochondrial gene expression as it not only generates the polycistronic transcripts,<sup>44</sup> without which there would be no expression of the mitochondrial genome, but also synthesizes the RNA primers necessary to initiate replication of mtDNA.<sup>45</sup> Despite the prokaryotic evolutionary origins of mitochondria, POLRMT is related to the single subunit bacteriophage T7 molecule rather than prokaryotic RNA polymerases.<sup>46</sup> Recent X-ray structures reveal that there is a unique helical domain in the molecule consisting of nine  $\alpha$ -helices of which there are two pairs that constitute PPR motifs.<sup>47</sup> The conserved residues within these PPR domains form part of the helix-turn-helix fold that interacts with the N-terminal domain, which is necessary for function.<sup>47</sup> The PPR domain has also been postulated to facilitate an interaction with LRPPRC, potentially linking transcription to subsequent post-transcriptional events acting on the newly synthesized mt-RNAs.<sup>46</sup> Other interaction partners of POLRMT include the transcription elongation factor of mitochondria (TEFM), an RNase-resistant association<sup>48</sup> and one of the mitoribosomal proteins, MRPL12.<sup>49</sup> This protein is part of the large subunit (LSU) and has been reported to exist both complexed with the LSU and as a free pool. It is the “free” MRPL12 that appears to bind POLRMT to activate transcription.<sup>50</sup> A recent report suggests that in addition to its role in transcription, POLRMT may also assist in maturation of the small ribosomal subunit (SSU).<sup>51</sup> The authors propose that by interacting with mitochondrial transcription factor B1 (h-mTFB1) in the SSU, POLRMT supports the dimethylation activity of h-mTFB1 on the 12S mt-rRNA, adding a quality control step to SSU and mitoribosome biogenesis.<sup>51</sup>



**Figure 1.** Cartoon depicting LRPPRC, a subset of its interacting partners, and processes in which it is involved. LRPPRC has a number of interactions that facilitate mitochondrial gene expression. It has also been reported to be involved in other cellular processes. The size of the arrow between LRPPRC and the surrounding balloons, together with the intensity of the balloon color, is an indicator of the amount of literature supporting these connections.

### MRPS27

The small subunit of mammalian mitoribosomes comprises ~30 polypeptides, almost half of which do not have eubacterial orthologs.<sup>52</sup> One of these is MRPS27 and it is a PPR protein. Investigation into this protein predicted that it contains six PPR domains but neither siRNA depletion nor overexpression of the protein were reported to affect levels of mature mt-RNAs. The authors analyzed the levels of other MRPs in the absence of MRPS27, but despite these appearing unaffected there was an apparent decrease in the efficiency of mt-translation with a more pronounced effect on synthesis of COXI and COXII.<sup>53</sup> Low-resolution cryo-EM studies had defined the structure of a bovine mitoribosomal preparation to approximately 10 Å.<sup>54</sup> Although this has resolved many questions about the differences between mammalian mitoribosomes and ribosomes from eubacteria and the eukaryotic cytosol, the definition remains too low to assign positions to each component polypeptide. Consequently, it is difficult to know exactly where in the mitoribosome this subunit resides. This raises again the importance of producing a high-resolution structure for the mammalian mitoribosome, which would also need to be demonstrated to function in vitro.

### PTCD1, 2 and 3

A cluster of three pentatricopeptide domain (PTCD)-containing proteins, namely PTCD1, PTCD2, and PTCD3, also affect mitochondrial translation. So far these have received less attention than the previous examples. PTCD1 was noted as sharing homology with a *Neurospora crassa* protein that acts as an assembly factor for complex I of the respiratory chain.<sup>55</sup> The human PTCD1 has been shown to be a mitochondrial matrix protein, with eight predicted PPR domains. It appears to have RNA-binding activity as it was

seen to associate with both mt-tRNA<sup>leu(UUR)</sup> and mt-tRNA<sup>leu(CUN)</sup> transcripts, and also uncleaved precursors containing either of these sequences.<sup>56</sup> Analysis indicates that PTCD1 regulates levels of mt-tRNA<sup>leu</sup> and that manipulation of PTCD1 by siRNA depletion results in increased steady-state of both mt-tRNA<sup>leu</sup> species. In an analogous fashion, overexpression of PTCD1 resulted in decreased mt-tRNA<sup>leu</sup> levels. However, neither depletion nor overexpression changed the overall levels of mature mt-mRNAs or mt-rRNAs. PTCD1 depletion was accompanied by a modest increase in expression of mt-encoded complex I and IV subunits, but only increased activity for complex IV.<sup>56</sup> Subsequent work by the same group has shown that PTCD1 may play a role in processing of the polycistronic RNA. Depletion of PTCD1 had a modest effect on increasing the number of partially processed intermediates. Conversely, overexpression of PTCD1 reduced the number of precursor RNA species. These effects were most apparent on the *MTND4-5* and *tRNA<sup>Ala</sup>-MTCO1* species.<sup>57</sup> Immunocapture of the mitoribosome via a large subunit protein, ICT1, revealed a number of interacting proteins. As mentioned above, this included LRPPRC, it also included PTCD1 but there has been no further investigation into this interaction.<sup>39</sup>

PTCD2 shares 17.8% identity with MRPS27 but investigations performed using a mouse model suggest that their functions are quite different.<sup>55</sup> The mouse PTCD2 was believed to have a single PPR motif, whereas the human has now been shown to have five.<sup>30</sup> RNA from numerous mouse tissues was analyzed revealing that there are varying levels of PTCD2 expression with highest levels in heart, liver and kidney.<sup>55</sup> A mouse model with a gene disruption in PTCD2 was then generated and the respiratory chain activities from these same tissues were analyzed in controls and mice with the PTCD2 disruption. There was a clear defect in heart for complex III and combined complex I+III activities, with a modest increase in complex IV in liver. Northern analysis indicated that levels of the unprocessed precursor encoding cytochrome *b* were elevated in the PTCD2 mutant mouse tissues compared with controls. Commensurately, the levels of fully processed *MTCYB* transcript were reduced. Consistent with this observation, subsequent western blotting of mitochondrial extracts indicated that as a consequence there were lowered levels of cytochrome *b* protein. The authors conclude that although PTCD2 disruption decreases complex III of the respiratory chain exclusively, it did seem to correlate with increased mitochondrial mass in liver, with mild but consistent alterations in cristae structure.<sup>55</sup> A surprising observation relating to PTCD2 is that it has been identified as a target for autoantibodies in neurodegenerative disease and is used as a biomarker for Alzheimer disease (AD).<sup>58</sup> Moreover, it appears that on western blot examination of brain from Alzheimer patients compared with controls, there are elevated levels of PTCD2 in the cerebral cortex of AD patients.<sup>59</sup>

In a similar fashion to PTCD1, PTCD3 has an ortholog that is an OXPHOS assembly factor, but in this case it is the ortholog of a plant protein that helps assemble complex V.<sup>55</sup> Human PTCD3 is predicted to contain 15 PPR domains, and as with PTCD2, its mRNA expression varies between tissues.<sup>30,60</sup> This protein was initially identified as a mitochondrial RNA-binding protein with some similarity to PPR proteins.<sup>61</sup> This has subsequently

been designated PTCD3. Consistent with a role in RNA binding and mitochondrial gene expression, PTCD3 was found associated with the mt-rRNA from the small ribosomal subunit but not other mt-rRNAs. Although it was found to associate with the SSU, the loss of PTCD3 did not appear to affect mitoribosome formation. Neither depletion nor overexpression affected mt-mRNA levels, implying that PTCD3 does not function in either mRNA processing or stability. Depleting PTCD3 in 143B osteosarcoma cells, however, did have a general effect on mitochondrial protein synthesis. This was decreased as was oxygen consumption and activities of complexes III and IV.<sup>60</sup> Haque et al. also found PTCD3 to be associated with the mt-SSU via the C terminus of mtIF3.<sup>62</sup> Reflecting this involvement in mt-translation PTCD3, as for PTCD1, was found to be a strong interactor with the mitoribosome<sup>39</sup> and also mitochondrial ribosome recycling factor.<sup>63</sup> A further substantiation of its role in RNA metabolism is the observation that it is found in association with mitochondrial RNA granules that are found in the matrix. In addition to PTCD3, these contain nascent mt-RNA, RNase P, and GRSF1.<sup>64</sup> As an unexplained but interesting aside, PTCD3 was also found in a different immunocapture experiment. Here, the aim was to find interaction partners of transcription elongation factor of mitochondria (TEFM) and, as mentioned above, another PPR protein, POLRMT, was identified. Unlike the POLRMT interaction, PTCD3/TEFM contact was lost when the sample was treated with RNase.<sup>48</sup> These various associations with protein components involved in different aspects of mt-RNA metabolism suggest there may yet be undiscovered roles for PTCD3.

### MRPP3

Last to be identified but not least of the human PPR proteins is mitochondrial RNase P protein 3 (MRPP3). This protein has been identified as a subunit of the mammalian mitochondrial RNase P complex.<sup>65</sup> As mentioned briefly earlier, human mtDNA is transcribed into long polycistronic transcripts that need to be cleaved into their constituent RNA species, separating the mt-tRNAs, from the -rRNAs and -mRNA species. According to the tRNA punctuation model first hypothesized by Ojala et al.,<sup>66,67</sup> the distribution of mt-tRNAs in these polycistronic units means that after their excision, the vast majority of the remaining RNAs are ready to be matured.<sup>66,67</sup> After many years of controversy, RNase P, the major enzyme complex responsible for part of this endonucleolytic activity, was clarified as consisting of only three proteins and no RNA component,<sup>65</sup> although a second, RNA-containing mitochondrial RNase P activity, has been described.<sup>68</sup> One of these three proteins was KIAA0391 and has since been renamed MRPP3, for mitochondrial RNase P protein 3. As with most PPRs, there is no crystal structure for MRPP3; however, the structure of the protein-only RNase P, a plant equivalent, has recently been published.<sup>6</sup> Although from a different organism, the structural information on the PPR element, PRORP1, may provide insight into how MRPP3 functions.<sup>69</sup> Here the metallonuclease region of the C terminus appears to be evolutionarily conserved and harbor the catalytic domain.<sup>69</sup> Also within the catalytic region are

four conserved residues that constitute a zinc-binding pocket.<sup>70</sup> This zinc binding is proposed to stabilize the structure to facilitate binding of the tRNA to PRORP1.<sup>6</sup> Consistent with other PPR proteins is the RNA-binding activity and the three tandem PPR motifs,<sup>30,50</sup> which in PRORP1 are N-terminal to the catalytic domain.<sup>6,69,70</sup> As mentioned above, MRPP3, together with MRPP1 as part of RNase P complex, were recently found in mitochondrial RNA granules that form discrete foci within the mitochondrial matrix. Perhaps MRPP3 is in these granules so that in addition to its role in mt-tRNA cleavage, or in concert with other as-yet-undiscovered PPR proteins, it can facilitate the endonucleolytic release of open reading frames such as *RNA14* that are not flanked on both sides by mt-tRNAs?

Cells lacking mtDNA ( $\rho^0$ ) would not be predicted to need proteins responsible for mt-RNA metabolism. This has been reported for a number of PPR proteins where downregulation of LRPPRC, PTCD1, PTCD2, and PTCD3 was observed,<sup>25</sup> and also ICT1, MRPL3, and EraL1 (authors' unpublished observations) confirming their importance in mitochondrial gene expression. Further, changes in one PPR protein seem to affect a subset of the others as mouse embryonic fibroblasts carrying a

LRPPRC mutation displayed an unanticipated increase in levels of PTCD2 and MRPS27.<sup>25</sup>

Analysis of the human genome has arguably revealed the entire set of protein-coding genes; however, the complexities of predicting PPR domains may have masked our ability to determine the complete set of PPR proteins. Even assuming that we do have a limited number of PPR proteins and they have all been identified, perhaps this is because in humans, mtRNA requires fewer manipulations and PPR proteins perform multiple functions. This is consistent with the increasing number of interacting proteins that are being found to associate with the known PPRs. If this is the case, then we can conclude that there are secrets about human PPR interactions and functions that are still left to be uncovered.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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