Conclusions and Future Scope of Work
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In the present study, we have demonstrated the existence of an LdUrm1-LdUba4 conjugation pathway in the human protozoan parasite *Leishmania donovani* including the enzymatic steps involved in the pathway viz activation by LdUba4 (E1) and conjugation with substrate proteins eventually. However, the E2 and E3 enzymes characteristic to Ub and Ubiquitin like molecules (Ubls) were not identified. Modification and/or alterations in the expression of LdUrm1 showed impaired growth underlining the importance of LdUrm1 mediated protein modifications in *Leishmania* growth and differentiation. Importantly, we identified and established that LdUrm1 was localized as a unique puncta near the flagellar pocket in the basal body which is the point of origin of the early endosomes in *Leishmania*. Moreover, the substrate proteins of LdUrm1 in *L. donovani* also appeared to be associated with the early endosome based on our MS analysis in both the stages of the parasite. However, the E1 enzyme for LdUrm1 was distinctly cytoplasmic in location. To our knowledge this is the first demonstration of a trypanosomatid Ubl pathway that is associated with early endosome and highlights its significance from heme uptake point of view which is one of the critical factor required for smooth operation of *Leishmania* metabolic machinery.

Earlier studies have showed that the Urm1 pathway is widely conserved in mammals, nematode and other multicellular organisms including unicellular eukaryotes such as yeast unlike other Ubls that are not found in unicellular eukaryotes, hence it is believed to be a molecular fossil amongst the different Ubls [Furukawa et al., 2000]. Our first of a kind demonstration of LdUrm1 pathways in trypanosomatid parasites, unicellular eukaryotes that emerged as early eukaryotic organisms indicated the ancient origin of this pathway. In trypanosomatid parasites, our analysis showed that the Ubls such as Ub, SUMO and Ufm1 appear to have one or more aminoacid residues after the C-terminal glycine that is needed to be processed before conjugation however LdUrm1 existed in pre-processed form with an exposed C-terminal di-Glycine motif suggesting absence of a processing protease. Moreover it also suggested that the C-terminal di-Glycine motif is crucial to subsequent conjugation of LdUrm1 to its target substrates. This is characteristically similar to apicomplexan parasites such as *Plasmodium* contain Ubls (Atg8) that terminate in a C-terminal Glycine indicating the absence of processing prior to conjugation [Ponder et al., 2007]. This structural diversity among different parasites might be a result of adaptations that parasites have undergone in their respective environments during their complex life cycles. Similar to humans and yeast, LdUrm1 had a specific E1-like (LdUba4) enzyme for activation however no E2 and E3 like
enzymes were identified. The activation process was found to be energy dependent that utilizes ATP. LdUrm1 was found to be very closely related to the structure of TbUrm1 and the structural and functional similarities amongst the two proteins was determined by homology modelling.

We further demonstrated that the *L. donovani* Urm1- Uba4 conjugation system can be reconstituted *in vitro* using recombinant proteins. A high molecular weight conjugate corresponding to *Ld*Urm1- LdUba4 adduct was observed by the enzymatic reactions of LdUba4 (E1-like), LdUrm1 with a free C-terminal glycine residue and ATP. The formation of a conjugate corresponding to LdUrm1- LdUba4 adduct was also seen *in vivo* using Co-IP methods that subsequently supported the hypothesis that the LdUrm1- LdUba4 conjugation pathway exists within *L. donovani*. Similarly, protein interactions between LdUrm1 and LdUba5 showed the conservation of E1-like activity in *L. donovani* parasites, thereby demonstrating the importance of E1 activity in the *Leishmania* Urm1 conjugation pathway.

In addition to above we also found that expression of non-conjugatable LdUrm1 (LdUrm1\(\Delta G\)) in *L. donovani* resulted in depleted parasite growth which suggested that LdUrm1 despite a housekeeping gene with constitutive expression is important to *Leishmania* biology.

Urm1 pathway has not been assigned any specific biological function in any organism so far however a number of substrates with diverse functions are known. These included (i) Ahp1, an alkyl hydroperoxide reductase with a role in oxidative stress protection in yeast [Goehring *et al.*., 2003]; (ii) Tot1/YLR384C10, an elongator subunit [Fichtner *et al.*., 2003]; (iii) ATPBD3, an uncharacterized Urm1 dependent enzyme required for thiolation of certain anticodon nucleosides [Schlieker *et al.*., 2008]; (iv) a functional crosslink with TOR (target of rapamycin) signaling pathway [Leidel *et al.*., 2009]; (v) thiolation of wobble uridine u34 of cytosolic tRNAs [Nakai *et al.*, 2008]. The components of this pathway involve URM1, UBA4, NFS1, NCS2, NCS6 and YOR251C in yeast [Bjork *et al.*, 2007; Nakai *et al.*, 2008; Dewez *et al.*, 2008; Huang *et al.*, 2008; Leidel *et al.*, 2009]. These findings suggested that Urm1 takes up a range of molecular functions across different species. Therefore, we employed MALDI-TOF/MS tool to identify the putative interacting targets substrates of LdUrm1 in *L. donovani*. Our MS results showed that LdUfm1 is potentially conjugated to the Rab-like GTPase activating protein, Ras-related protein Rab-5 protein that localize the early endosome. It has been established earlier that *L. donovani* specific Rab5b localizes to early endosome [Marotta *et al.*, 2006]. Moreover, *Leishmania* endocytoses hemoglobin (Hb) through a specific receptor
located in the flagellar pocket [Singh et al., 2003; Krishnamurthy et al., 2005] and bound Hb is rapidly internalized into discrete Rab5 positive early endosomal compartment and subsequently targeted to the lysosomal compartment in a Rab7 dependent way [Singh et al., 2003; Patel et al., 2008]. Taken together, our identification of Rab5 and Rab GTPase as LdUrm1 target clued possible role of LdUrm1 in early endosome mediated heme internalization in Leishmania. Additionally, we also identified a hypothetical protein with Leucine rich repeats (LRR) that showed homology to internalin A, a protein that belonged to Li. monocytogenes internalin family. Intriguingly, our identification of a putative E3 ubiquitin-protein ligase RSP5, HECT domain containing protein in our MS data also raised the possibility of the existence of an E3 ligase enzyme in LdUrm1 conjugation pathway. To our knowledge, this is the first report that demonstrates the presence of complete LdUrm1- LdUba4 conjugation pathway along with its substrates in the early endosome of Leishmania.

Future scope of work

Demonstration of the existence of early endosome associated LUrm1-LdUba4 conjugation in unicellular trypanosomatid parasites offers peculiar possibilities to explore the importance of this pathway in the context of its pathogenesis and host-parasite interactions. Importantly, the ubiquitin-dependent proteolysis system (UPS) is increasingly being recognized as a promising therapeutic pathway in the treatment of cancer hematological malignancies have been treated successfully using proteasome inhibitors [Elridge et al., 2010]. Deubiquitinases, the key effectors of Ubiquitin Proteasome System and intracellular signalling cascades, in addition to Ub ligases are emerging as important targets for potential anticancer therapies because of their narrow substrate specificity [Sacco et al., 2010, Goldenberg et al., 2010]. Identification of Urm1 mediated protein modification pathways in Leishmania, with its distinct subset of substrate proteins associated with early endosome as demonstrated in this report strengthens our understanding of Leishmania biology and subsequently presents a new set of specific targets unique to a conjugation pathway which may be exploited in near future for designing novel drug therapies against this human pathogen.