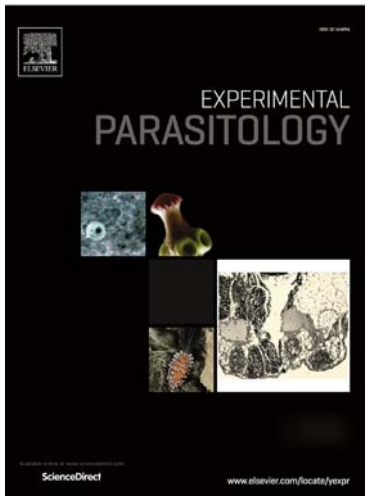


Cytosolic *Trypanosoma cruzi* nucleoside diphosphate kinase generates large granules that depend on its quaternary structure

Claudio A. Pereira, Chantal Reigada, M. Sayé, Fabio A. Digirolamo, Mariana R. Miranda

PII: S0014-4894(14)00098-8
DOI: http://dx.doi.org/10.1016/j.exppara.2014.04.009
Reference: YEXPR 6862

To appear in: *Experimental Parasitology*

Received Date: 28 November 2013
Revised Date: 28 March 2014
Accepted Date: 17 April 2014

Please cite this article as: Pereira, C.A., Reigada, C., Sayé, M., Digirolamo, F.A., Miranda, M.R., Cytosolic *Trypanosoma cruzi* nucleoside diphosphate kinase generates large granules that depend on its quaternary structure, *Experimental Parasitology* (2014), doi: http://dx.doi.org/10.1016/j.exppara.2014.04.009

Cytosolic *Trypanosoma cruzi* nucleoside diphosphate kinase generates large granules that depend on its quaternary structure

Claudio A. Pereira[a], Chantal Reigada[a], SayéM.[a], Fabio A. Digirolamo[a] and Mariana R. Miranda[a]*

[a]*Laboratorio de Biología Molecular de Trypanosoma cruzi (LBMTC), Instituto de Investigaciones Médicas Alfredo Lanari, Universidad de Buenos Aires and CONICET, Buenos Aires, Argentina*

Correspondingauthor: M. Miranda, IDIM, Combatientes de Malvinas 3150, (1427) Bs. As. Argentina. Phone: 5411-4514-8701; FAX: 5411-4523-8947; E-mail address: mirandamariana@gmail.com

Abstract

Nucleoside diphosphate kinase (NDPK) is a key enzyme in the control of cellular concentrations of nucleoside triphosphates, and has been shown to play important roles in many cellular processes. In this work we investigated the subcellular localization of the canonical NDPK1 from *Trypanosoma cruzi* (TcNDPK1), the etiological agent Chagas´ Disease, and evaluated the effect of adding an additional weak protein-protein interaction domain from the green fluorescent protein (GFP). Immunofluorescence microscopy revealed that the enzyme from wild-type and TcNDPK1 overexpressing parasites has a cytosolic distribution, being the signal more intense around the nucleus. However, when TcNDPK1 was fused with dimeric GFP it relocalizes in non-membrane bounded granules also located adjacent to the nucleus. In addition, these granular structures were dependent on the quaternary structure of TcNDPK1 and GFP since mutations in residues involved in their oligomerization dramatically decrease the amount of granules. This phenomenon seems to be specific for TcNDPK1 since other cytosolic hexameric enzyme from *T. cruzi,* such as the $NADP^+$-linked glutamate dehydrogenase, was not affected by the fusion with GFP. In addition, in parasites without GFP fusions granules could be observed in a subpopulation of epimastigotes under metacyclogenesis and metacyclic trypomastigotes. Organization into higher protein arrangements appears to be a singular feature of canonical NDPKs; however the physiological function of such structures requires further investigation.

Keywords: *Trypanosoma cruzi*; Nucleoside diphosphate kinase; granules; protein arrangement; oligomerization.

1. Introduction

Nucleoside diphosphate kinases (NDPK; EC 2.7.4.6) have been characterized as a large family of conserved proteins that synthesize nucleoside triphosphates from nucleoside diphosphates and ATP(Veron, et al., 1994). The enzyme appears to be expressed in all organisms and its amino acid sequence is highly conserved between prokaryotes and eukaryotes (Bilitou, et al., 2009). Canonical eukaryotic NDPKs are non-covalently bonded homohexamers of 17-kDa subunits arranged into a trimer of dimers. The hexameric structure is important for protein stability and essential for enzyme activity(Janin, et al., 2000). One of the main functions of the NDPKs is the maintenance of intracellular nucleoside triphosphate pools. The enzyme has also additional roles in DNA-processing, apoptosis and many other regulatory processes (Choudhuri, et al., 2010, Hippe, et al., 2011, Kolli, et al., 2008, Postel, 2003, Postel and Abramczyk, 2003). Since they are multifunctional proteins the interest in NDPKs has increased substantially in recent years. In accordance with their functions, they are localized all along the cellular compartments among the different organisms, i.e. cytoplasm, nucleus, mitochondria, flagellum and cell membrane (Anderca, et al., 2002, Hammargren, et al., 2007, Hunger-Glaser, et al., 2000, Patel-King, et al., 2004, Yoshida and Hasunuma, 2006, Zhang, et al., 1995).

Trypanosoma cruzi is a protozoan parasite and the causative agent of Chagas`s Disease, an illness affecting millions of people in America. In contrast to mammals, all protozoan parasites, including *Trypanosoma*, are unable to synthesize purines de novo and therefore rely upon the salvage of these compounds from host cells where nucleoside diphosphate kinases are involved (Carter, et al., 2001). In a previous work we reported the presence of a canonical NDPK in *T. cruzi*, called TcNDPK1 (N1), in addition to other three divergent isoforms; TcNDPK2-4 (Miranda, et al., 2008). TcNDPK2 has been characterized as a tubulin-associated enzyme having cytosolic

and cytoskeleton localization (Miranda, et al., 2011). We also demonstrated that N1 has wide range nuclease activity (Miranda, et al., 2008) as was also observed for many canonical NDPKs from different organisms (Hammargren, et al., 2007, Kumar, et al., 2005, Levit, et al., 2002, Postel, 1999). Additionally, *T. brucei* homologue was located in the nucleus of the parasites (Hunger-Glaser, et al., 2000), suggesting possible roles in nuclear processes. In a recent work it was also determined that N1 is secreted into the host inside lipidic vesicles (Bayer-Santos, et al., 2013) giving new possible functions in pathogenesis and virulence, as occurs in *Leishmania* and *Mycobacterium* (Chopra, et al., 2003, Kolli, et al., 2008, Saini, et al., 2004).

As was mentioned above, subcellular localization of these enzymes have been extensively studied by different approaches, however, fusion with fluorescent proteins led us to unexpected results. Our experience working with the recombinant N1 as well evidences of recombinant NDPKs from other organisms, indicate that the enzyme may be present in the form of protein aggregates (Lascu, et al., 2000).In this way, the addition of protein tags to NDPKs may affect their properties, especially if these tags are dimmers like GFP. In the present work we investigated the subcellular localization of the canonical NDPK1 from *Trypanosoma cruzi* and evaluated the effect of fusing such an oligomeric enzyme to a dimeric GFP. We observed a cytosolic localization for the endogenous enzyme which relocalizes in granular structures when it was fused to GFP. Such granular structures might suggest implications in physiological processes.

2. Materials and Methods

2.1 *Cell cultures and generation of transgenic parasites.*

Epimastigotes of the MJ-Levin (discrete typing unit I, DTU I) and Y (DTU II) strains (Bouvier LA, 2013, Zingales, et al., 2009) were cultured at 28 ℃ in plastic flasks (25 cm^2), containing 5 mL of BHT medium (started with 10^6 cells per milliliter) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL

streptomycin (Camargo, 1964). Transgenic parasites were obtained by electroporation of $3x10^8$ cells with 50 µg of plasmidic DNA in PBS buffer with 0,1 mM $CaCl_2$ and 0,5 mM $MgCl_2$ in a Biorad Gene Pulser Xcell (400 V, 500 µF). The selection was made with 500 µg/ml of G418 and the parasites were then maintained in 200 µg/ml of the drug. In the cases of co-transfection experiments and *GDH*::*GFP* (*GDH*::*G*) construct, transient expression was evaluated 24 h after electroporation. Trypomastigotes and amastigotes were obtained from epimastigotes of the Y strain overexpressing the NDPK1 fused to the epitopes HA and EEF (N1-HE) as previously described (de Camara Mde, et al., 2013). Metacyclic trypomastigotes were generated by treating epimastigotes in TAU-P medium followed by purification through a DEAE column.

2.2 *Antibodies*.

Balb/C mice of 45 days of age received 3 intraperitoneal administrations of 10 µg of recombinant His-tag N1 every 15 days, treated with 0.05 % of hydrogen peroxide and mixed with an equal volume of Freund´s adjuvant. Serum containing anti-N1 antibodies was obtained from the blood of the mice and used in the assays.

2.3 *Cloning*.

The genes of TcNDPK1 (*N1* TritrypDB: TcCLB.508707.200) and the $NADP^+$-linked glutamate dehydrogenase (*GDH* TritrypDB: TcCLB.508111.30) were obtain by PCR from the pGEM-T Easy-*N1* vector previously generated (Miranda, et al., 2008) and *T. cruzi* genomic DNA respectively, with the primers N1FF 5´ TCTAGAATGACCAGTGAGCGTACCTTC 3´, N1FR 5´GTCGACTGCAGACTCGTAGACCTGCTT 3´, GDHF 5´ TCTAGAATGACCTCGCTTTGGCCTTT 3´, GDHR 5´GTCGACAACTACGCCAAGACCCTTCATG 3´. The mutations in the N1 gene were generated by Gene Splicing by Overlapping Extension PCR. The mutation *N1P95S* was obtained with the primers N1P95SF 5´CGCAACAAACAGTGCTGACTCAC 3´, N1P95SR 5´ GTGAGTCAGCACTGTTTGTTGCG 3´, N1∆4CR 5´

GTCGACGACCTGCTTGACGGAATGTGA 3´ and N1FF; the mutation *N1H117N* was obtained with the primers N1H117NF 5´ AACGTGTGCAATGGCTCTGAC 3´, N1H117NR 5´ GTCAGAGCCATTGCACACGTT 3´, N1FF and N1FR. All the PCR products were cloned in the pGEM-T Easy vector (Promega), sequenced and sub-cloned in the pTREX-L-*eGFP* vector (pT-L-*G*), described in (Bouvier LA, 2013), in the XbaI and SalI recognition sites (underlined text). The final constructs contained the genes fused with the *GFP* together with the *HA* and *EEF* epitopes (*H* and *E*, respectively), so a HindIII digestion, filling and ligation was required in order to suppress those epitopes (for more details see (Bouvier LA, 2013). In the case of the *N1*::*mCherry* fusion a pTREX-L-*mCherry* vector (pT-L-*Ch*) was used and the final construct contained both epitopes. The *N1*::*HE* fusion was obtained through cloning into the XbaI-SalI sites from an intermediate pT-L vector. The pT-*N1* construction was generated by subcloning the N1 gene from the pGEM-Teasy-*N1* vector in the EcoRI site of the pTREX vector (Vazquez and Levin, 1999). The non-dimeric eGFP (*mG*) was generated as previously reported (A206K variant) (von Stetten, et al., 2012) from the pT-L-*G* vector using the primers mGF 5´CACCCAGTCCAAGCTGAGCAAAG 3´, mGR 5´ CTTTGCTCAGCTTGGACTGGGTG 3´, GF 5´ ACCATCTTCTTCAAGGACGA 3´ and GR 5´ GGCTGTTGTAGTTGTACTCC 3´. All the final constructions were checked by sequencing.

2.4 *Fluorescence Microscopy.*

Wild type epimastigotes and transgenic parasites (epimastigotes andtrypomastigotes) overexpressing the constructs pT-*N1* or pT-L-*N1::HE* were collected, washed twice with PBS and settled for 20 min onto poly-L-lysine coated coverslips. Then, parasites were fixed at room temperature for 20 min with 4% formaldehyde in PBS, permeabilized with cold methanol for 5 min and rehydrated in PBS for 15 min. The samples were blocked with 1% BSA in PBS for 10 min and incubated with primary antibodies in blocking buffer (mouse anti-N1 serum 1/50 dilution, rat anti-HA (Roche

Applied Science) 1/500 dilution) for 45 min. After three washes, the parasites were incubated with fluorescent anti-mouse or anti-rat IgG (DyLight 488 both) at a dilution of 1/500 for 30 min, washed and mounted using Vectashield with DAPI (Vector Laboratories). In the case of transgenic parasites expressing GFP (G) or mCherry (Ch) fusions, they were settled and fixed as was mentioned above and mounted for direct fluorescence observation. For amastigotes immunofluorescence, VERO cells were grown on coverslips and infected. After 72 h post-infection, cells were fixed for 1 h with 4% formaldehyde, permeabilized with 0.2% triton in PBS for 10 min and followed as indicated above. Parasites were observed in an Olympus BX60 fluorescence microscope and the images were recorded with an Olympus XM10 camera. For phase contrast images a confocal fluorescent microscope Zeiss LSM50 was used. Images were processed using the ImageJ software v.1.42 and granule sizes were analyzed with the "particle analysis" plugin of the same program.

2.5 *Western Blots.*

Parasites were collected, counted in a hemocytometer chamber, washed with PBS and resuspended directly with cracking buffer 1X (50 mMTris-HCl buffer pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol and 0.01% bromophenol blue). A volume corresponding to $5x10^6$ parasites was run in 12-15% SDS-polyacrylamide gels (PAGE) and transferred onto a PVDF membrane. The membranes were blocked for one hour in 5% of nonfat milk in T-PBS (PBS-0.005% Tween20) and incubated with primary antibodies overnight in blocking buffer (mouse anti-N1 1/500 dilution, rabbit anti-GFP (Molecular Probes) 1/5000 dilution, rat anti-HA 1/4000 dilution, rabbit anti-GDH 1/5000 dilution). After three washes, the membranes were incubated with Peroxidase-conjugated anti-mouse, anti-rabbit or anti-rat antibodies diluted 1/5000. The proteins were reveled with Super Signal West Pico Chemiluminescent substrate (Pierce). For native electrophoresis, parasites in Tris-HCl buffer 50 mM pH 6.8 were subjected to 4 cycles of freezing and thawing and centrifuged. The supernatant was mixed with

loading buffer 5X (50 mMTris-HCl buffer pH6.8, 50% glycerol and 0.05% Bromophenol blue). The volume corresponding to $5x10^6$ parasites was run in 8% PAGE without SDS and transferred onto PVDF membranes.

2.6 *Digitonin extraction.*

Digitonin extraction was carried out as previously described (Miranda, et al., 2008). Briefly, epimastigote cells were washed twice and resuspended in 50 mM Tris–HCl buffer, pH 7.5, containing 0.25 M sucrose and 10 µM E64. Aliquots of 950 µl containing $6.5x10^8$ parasites were mixed with 50 µl of the same buffer containing increasing amounts of digitonin (0–5 mg/ml). After 2.5 min of incubation at room temperature, tubes were centrifuged at 16,100g for 2 min. Pellets were resuspended in the same buffer and equal volumes of supernatants and pellets were analyzed by Western Blot.

2.7 *Transmission electron microscopy*.

10 ml of exponentially growing parasites expressing the N1-GFP (N1-G) or GFP proteins were washed in PBS and fixed with 0.5 % glutaraldehyde-2% paraformaldehyde in PBS for 24 h. The cells were centrifuged and dehydrated in increasing acetone series and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Philips EM-301 transmission electron microscope at the “Centro de Microscopías Avanzadas” of the University of Buenos Aires.

2.8 Nucleoside diphosphate kinase activity.

Parasites were collected, counted in a hemocytometer chamber, washed with PBS and resuspended in 100 mM Tris-HCl buffer pH 7.2. Protein extracts were obtained by 7 freezing and thawing cycles followed by centrifugation. The activity was measured as previously reported (Miranda, et al., 2008).

3. Results

3.1 N1 has a cytosolic localization

In a previous work we determined that about 80% of the total NDPK activity of *T. cruzi* epimastigotes was in the soluble fractions of digitonin extractions at low concentrations of the detergent (Miranda, et al., 2008). This cytosolic activity is probably due to the contribution of different isofoms. To further study the subcelullar localization of N1 in epimastigotes, we made specific antibodies against the protein and carried out immunofluorescence microscopy assays. We observed a diffuse staining throughout the cytosol being more intense around the nucleus (Figure 1A, WT). Western Blot analysis using fractions from slight digitonin extractions, showed that N1 is released in a similar way as the cytosolic marker $NADP^+$ -linked glutamate dehydrogenase (GDH)(Barderi, et al., 1998) (Figure 1B). In addition, we generated parasites overexpressing N1 which had a six fold increase in the NDPK activity and higher enzyme levels than controls (Figure 2A, lanes 1-2). The same result as wild-type cells was observed in immunofluorescence images from N1-overexpressing parasites, thus concluding that the enzyme is cytosolic and its overexpression did not affect the localization (Figure 1A, N1).

3.2 N1 fused with dimeric GFP relocalizes into protein granules

To study the enzyme dynamics *in vivo*, N1 gene was fused with the dimeric GFP at the C-terminus (*N1*::*G*) and then overexpressed in epimastigotes of *T. cruzi*. Transgenic parasites were analyzed for N1 expression by Western Blot (Figure 2A, lane 7; 2C, lane 1) and also observed at the fluorescence microscope. Interestingly, the fusion protein was localized in a single granule inside de cell, positioned, in most cases, adjacent to the nucleus, while control parasites transfected with GFP conserved the cytosolic localization (Figure 3A, N1-G and GFP). The observed granules all along the parasites population were of different size probably due to the heterogeneity in the expression levels of the fusion protein. The majority of this granules occupied 5 to 15 % of the cell body; however, some of them were larger, reaching up to 50% of the parasite as was observed under differential interference contrast microscopy (Figure

3A, N1-G). Moreover, the reciprocal N-terminal fusion (*G::N1*) generates the same pattern, suggesting that the observed phenotype was not a consequence of blocking an unknown localization signal (data not shown). These transgenic parasites contain an increase of about 3-fold in NDPK activity related to the GFP expressing control, suggesting that the fusion does not affect the activity and the hexamer conformation needed for the enzyme catalysis. In addition, analysis of the parasites by transmission electron microscopy showed that the granules were solid and non-membrane bounded particles (Figure 3B), indicating that the N1-G protein does not localize in any organelle and seems to be aggregated or assembled into a protein arrangement.

3.3 Granule formation is specific for N1 and depends on the oligomeric structures of N1 and GFP

Taking into account the obtained results, we then evaluated if the observed granules were also formed when N1 was fused with mCherry, a fluorescent monomeric protein similar to GFP (*N1*::*Ch*), or with low-molecular weight epitopes such as haemoglutinin (HA) and the tripeptide EEF (*N1*::*HE*). After parasites' transfection and selection they were checked for the correct expression of the fusion proteins by Western Blot (Figure 2A, lanes 3-4) and analyzed under fluorescence microscopy. In both cases, no granular structures were observed. The chimeric proteins were homogeneously distributed in the cytosol (Figure 4A, N1-HE and N1-Ch) suggesting that granule formation depends on the oligomeric state of the fused moiety and not on other protein property such as molecular weight. So, a non-dimeric GFP (A206K)(von Stetten, et al., 2012) was used in additional localization assays and, as was expected, no granules were observed (Figure 4A, N1-mG).To further assess if the assembly of the subunits of N1 into the hexamer is implicated in the organization of such granules, we generated a new NDPK construct bearing a mutation in the Pro95 residue (P95S) and a deletion in the four carboxyl-terminal amino acids which were reported to destabilize the quaternary structure of the enzyme (Karlsson, et al., 1996). This mutant was fused with

the dimeric GFP, expressed in the parasites and analyzed by Western Blot for the correct expression and destabilization of the structure using denaturing and non-denaturing PAGE (Figures 2A, lane 6; 2B, lane 2; 2C lane 3). As expected, these cells had about 40% of the NDPK activity present in N1-G overexpressing epimastigotes. In these parasites, a partial granule formation, in addition to a cytoplasmic distribution, could be observed (Figure 4A, N1P95S-G) indicating that the hexameric structure of N1 is involved in the process. In addition, the fluorescence signal was also detected inside the nucleus, indicative of monomers permeation into this organelle. Supporting this observation, in a co-transfection experiment, where the N1-G and N1-Ch were co-expressed, the granules contain both proteins, possibly as a consequence of the hetero-multimerization of the fusion proteins (Figure 4B). In the same way, we tested an inactive N1 mutated in the catalytic histidine (H117N). The NDPK activity of these parasites was about 20% of the N1-G ones, confirming the lack of function. As is shown in Figure 4A (N1H117N-G), the chimeric protein formed the same granules as the wild type, suggesting that the enzymatic activity does not affect the granule formation. Additionally, we determined if this aggregation phenomenon is specific to the N1 or could also involve other multimeric enzymes when they are fused with dimeric GFP. The same approach used with N1 was applied to the cytosolic $NADP^+$ -linked GDH from *T. cruzi* (Barderi, et al., 1998), another hexameric enzyme, and we observed that its localization was not affected by the addition of the GFP and no granular structure was observed (Figure 4A, GDH-G).

Finally, taking into account that N1 granules might take place in other stages of the parasite, we investigated the localization of N1 in N1-HE expressing trypomastigotes (metacyclic and derived from infected cells) and intracellular amastigotes. Although the localization in these cells was cytosolic as in the epimastigote stage (Figure 5, rows A, B, C), in a minor subpopulation of metacyclic trypomastigotes and in epimastigotes

under metacyclogenesis, obtained from parasites in stationary phase of culture and from TAU-P treatment, similar granules could be detected (Figure 5, rows D, E, F, G).

4. Discussion

In this work we investigated the subcellular localization of the canonical NDPK from *Trypanosoma cruzi* by immunofluorescence techniques and by expression fused with different polypeptides. Even though under *in vitro* epimastigote culture conditions N1 has a cytosolic localization, it was able to form protein granules when it was fused with a dimeric protein. These granules were dependent on the oligomeric state of the NDPK and the fluorescent protein. It was postulated that the fusion of these enzymes with tightly associated dimers, such as glutathione *S*-transferase, might generate aggregates by affecting it physical properties and it interaction with other proteins (Lascu, et al., 2000). A similar event seems to be occurring with the dimeric GFP *in vivo*. However, what is really interesting is that the aggregation pattern does not happen with another hexameric enzyme, for example the cytosolic $NADP^+$-linked GDH, nor with divergent NDPKs from *T. cruzi* or humans (Milon, et al., 2000, Miranda, et al., 2011). In addition, the fusion with GFP is currently one of the most common approaches used to study the subcellular localization of any protein and there is no report of such granule formation, including dimers and tetramers from different organisms (Cho, et al., 2006, Shashidharan, et al., 1999, Tjhin, et al., 2013). In an earlier work, other authors studying the subcellular localization of the human canonical NDPK A/B observed enzyme aggregates (Bosnar, et al., 2009) similar to N1-G ones. All these observations suggest that the formation of granules could be a particular feature of canonical NDPKs and its assembling into higher arrangements could be part of its catalytic mechanism of action. The precise localization besides the nucleus supports this hypothesis. In addition, the granules were formed under stress conditions in parasites without GFP fusions, such as epimastigotes under metacyclogenesis and

metacyclic trypomastigotes, thus resembling physiological conditions. Bosnar *et al* (Bosnar, et al., 2009) found that the human NDPK A carrying the inactivating point mutation H118N, did not develop aggregates. In accordance, it has been reported that recombinant NDPK B undergoes self-assembly into filaments *in vitro* in a nucleoside triphosphate dependent manner (Morin-Leisk and Lee, 2008). Such assembly could be someway related to the granules observed *in vivo*. Our results suggest that the granules are dynamic structures as the cytosolic N1-Ch was contained in the granules when it was co-expressed with N1-G. Additional observations indicate that changes in the intracellular ionic strength affect the granule stability, since parasites under hypo-osmotic and hyper-osmotic stresses generate delocalization of N1-G granules into multiple and smaller ones (data not shown).This behavior could be associated somehow to a polymerizing-depolymerizing process.

We hypothesize that the addition of a new protein-protein interaction domain increases the binding force resembling a reduction of the intermolecular distance, which might occur under physiological conditions.

All these evidences reflect new insights of NDPK enzymes which functions in physiological processes requires further investigations.

Acknowledgements

We thank Dr. Juan José Cazzulo (IIB-UNSAM) for kindly providing us anti-GDH antibodies and Dr. León A. Bouvier for the constructive advices he gave to us.This study was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 2010-0685 and PIP 2011-0263), Agencia Nacional de Promoción Científica y Tecnológica (FONCYT PICT 2008-1209, PICT 2010-0289 and PICT 2012-0559) and Fundación Bunge y Born 2012.C.A.P. and M.R.M. are members of the career of scientific investigator of CONICET (Argentina), C.R., and M.S.are research fellows from the same institution.

References

1. Anderca, M. I., Furuichi, T., Pinontoan, R., and Muto, S., 2002. Identification of a mitochondrial nucleoside diphosphate kinase from the green alga Dunaliella tertiolecta. Plant Cell Physiol 43**,** 1276-1284.
2. Barderi, P., Campetella, O., Frasch, A. C., Santome, J. A., Hellman, U., Pettersson, U., and Cazzulo, J. J., 1998. The NADP+-linked glutamate dehydrogenase from Trypanosoma cruzi: sequence, genomic organization and expression. Biochem J 330 (Pt 2)**,** 951-958.
3. Bayer-Santos, E., Aguilar-Bonavides, C., Rodrigues, S. P., Cordero, E. M., Marques, A. F., Varela-Ramirez, A., Choi, H., Yoshida, N., da Silveira, J. F., and Almeida, I. C., 2013. Proteomic analysis of Trypanosoma cruzi secretome: characterization of two populations of extracellular vesicles and soluble proteins. J Proteome Res 12**,** 883-897.
4. Bilitou, A., Watson, J., Gartner, A., and Ohnuma, S., 2009. The NM23 family in development. Mol Cell Biochem 329**,** 17-33.
5. Bosnar, M. H., Bago, R., and Cetkovic, H., 2009. Subcellular localization of Nm23/NDPK A and B isoforms: a reflection of their biological function? Mol Cell Biochem 329**,** 63-71.
6. Bouvier LA, C. M., Canepa GE., Miranda MR., and Pereira CA., 2013. Plasmid vectors and molecular building blocks for the development of genetic manipulation tools for Trypanosoma cruzi. PLoS One 8 (10)**,** e80217.
7. Camargo, E. P., 1964. Growth and Differentiation in Trypanosoma Cruzi. I. Origin of Metacyclic Trypanosomes in Liquid Media. Rev Inst Med Trop Sao Paulo 6**,** 93-100.
8. Carter, N. S., Landfear, S. M., and Ullman, B., 2001. Nucleoside transporters of parasitic protozoa. Trends Parasitol 17**,** 142-145.
9. Cho, J. I., Ryoo, N., Ko, S., Lee, S. K., Lee, J., Jung, K. H., Lee, Y. H., Bhoo, S. H., Winderickx, J., An, G., Hahn, T. R., and Jeon, J. S., 2006. Structure, expression, and functional analysis of the hexokinase gene family in rice (Oryza sativa L.). Planta 224**,** 598-611.
10. Chopra, P., Singh, A., Koul, A., Ramachandran, S., Drlica, K., Tyagi, A. K., and Singh, Y., 2003. Cytotoxic activity of nucleoside diphosphate kinase secreted from Mycobacterium tuberculosis. Eur J Biochem 270**,** 625-634.
11. Choudhuri, T., Murakami, M., Kaul, R., Sahu, S. K., Mohanty, S., Verma, S. C., Kumar, P., and Robertson, E. S., 2010. Nm23-H1 can induce cell cycle arrest and apoptosis in B cells. Cancer Biol Ther 9**,** 1065-1078.
12. de Camara Mde, L., Bouvier, L. A., Miranda, M. R., Reigada, C., and Pereira, C. A., 2013. Laboratory techniques to obtain different forms of Trypanosoma cruzi: applications to wild-type and genetically modified parasites. Folia Parasitol (Praha) 60**,** 406-410.
13. Hammargren, J., Salinas, T., Marechal-Drouard, L., and Knorpp, C., 2007. The pea mitochondrial nucleoside diphosphate kinase cleaves DNA and RNA. FEBS Lett 581**,** 3507-3511.
14. Hippe, H. J., Wolf, N. M., Abu-Taha, H. I., Lutz, S., Le Lay, S., Just, S., Rottbauer, W., Katus, H. A., and Wieland, T., 2011. Nucleoside diphosphate kinase B is required for the formation of heterotrimeric G protein containing caveolae. Naunyn Schmiedebergs Arch Pharmacol 384**,** 461-472.
15. Hunger-Glaser, I., Hemphill, A., Shalaby, T., Hanni, M., and Seebeck, T., 2000. Nucleoside diphosphate kinase of Trypanosoma brucei. Gene 257**,** 251-257.
16. Janin, J., Dumas, C., Morera, S., Xu, Y., Meyer, P., Chiadmi, M., and Cherfils, J., 2000. Three-dimensional structure of nucleoside diphosphate kinase. J Bioenerg Biomembr 32**,** 215-225.

17. Karlsson, A., Mesnildrey, S., Xu, Y., Morera, S., Janin, J., and Veron, M., 1996. Nucleoside diphosphate kinase. Investigation of the intersubunit contacts by site-directed mutagenesis and crystallography. J Biol Chem 271**,** 19928-19934.
18. Kolli, B. K., Kostal, J., Zaborina, O., Chakrabarty, A. M., and Chang, K. P., 2008. Leishmania-released nucleoside diphosphate kinase prevents ATP-mediated cytolysis of macrophages. Mol Biochem Parasitol 158**,** 163-175.
19. Kumar, P., Verma, A., Saini, A. K., Chopra, P., Chakraborti, P. K., Singh, Y., and Chowdhury, S., 2005. Nucleoside diphosphate kinase from Mycobacterium tuberculosis cleaves single strand DNA within the human c-myc promoter in an enzyme-catalyzed reaction. Nucleic Acids Res 33**,** 2707-2714.
20. Lascu, L., Giartosio, A., Ransac, S., and Erent, M., 2000. Quaternary structure of nucleoside diphosphate kinases. J Bioenerg Biomembr 32**,** 227-236.
21. Levit, M. N., Abramczyk, B. M., Stock, J. B., and Postel, E. H., 2002. Interactions between Escherichia coli nucleoside-diphosphate kinase and DNA. J Biol Chem 277**,** 5163-5167.
22. Milon, L., Meyer, P., Chiadmi, M., Munier, A., Johansson, M., Karlsson, A., Lascu, I., Capeau, J., Janin, J., and Lacombe, M. L., 2000. The human nm23-H4 gene product is a mitochondrial nucleoside diphosphate kinase. J Biol Chem 275**,** 14264-14272.
23. Miranda, M. R., Camara Mde, L., Bouvier, L. A., and Pereira, C. A., 2011. TcNDPK2, a Trypanosoma cruzi microtubule-associated nucleoside diphosphate kinase. Mol Biochem Parasitol 177**,** 152-155.
24. Miranda, M. R., Canepa, G. E., Bouvier, L. A., and Pereira, C. A., 2008. Trypanosoma cruzi nucleoside diphosphate kinase 1 (TcNDPK1) has a broad nuclease activity. Parasitology 135**,** 1661-1666.
25. Miranda, M. R., Canepa, G. E., Bouvier, L. A., and Pereira, C. A., 2008. Trypanosoma cruzi: multiple nucleoside diphosphate kinase isoforms in a single cell. Exp Parasitol 120**,** 103-107.
26. Morin-Leisk, J. and Lee, T. H., 2008. Nucleotide-dependent self-assembly of Nucleoside Diphosphate Kinase (NDPK) in vitro. Biochim Biophys Acta 1784**,** 2045-2051.
27. Patel-King, R. S., Gorbatyuk, O., Takebe, S., and King, S. M., 2004. Flagellar radial spokes contain a Ca2+-stimulated nucleoside diphosphate kinase. Mol Biol Cell 15**,** 3891-3902.
28. Postel, E. H., 1999. Cleavage of DNA by human NM23-H2/nucleoside diphosphate kinase involves formation of a covalent protein-DNA complex. J Biol Chem 274**,** 22821-22829.
29. Postel, E. H., 2003. Multiple biochemical activities of NM23/NDP kinase in gene regulation. J Bioenerg Biomembr 35**,** 31-40.
30. Postel, E. H. and Abramczyk, B. M., 2003. Escherichia coli nucleoside diphosphate kinase is a uracil-processing DNA repair nuclease. Proc Natl Acad Sci U S A 100**,** 13247-13252.
31. Saini, A. K., Maithal, K., Chand, P., Chowdhury, S., Vohra, R., Goyal, A., Dubey, G. P., Chopra, P., Chandra, R., Tyagi, A. K., Singh, Y., and Tandon, V., 2004. Nuclear localization and in situ DNA damage by Mycobacterium tuberculosis nucleoside-diphosphate kinase. J Biol Chem 279**,** 50142-50149.
32. Shashidharan, P., Chalmers-Redman, R. M., Carlile, G. W., Rodic, V., Gurvich, N., Yuen, T., Tatton, W. G., and Sealfon, S. C., 1999. Nuclear translocation of GAPDH-GFP fusion protein during apoptosis. Neuroreport 10**,** 1149-1153.
33. Tjhin, E. T., Staines, H. M., van Schalkwyk, D. A., Krishna, S., and Saliba, K. J., 2013. Studies with the Plasmodium falciparum hexokinase reveal that PfHT limits the rate of glucose entry into glycolysis. FEBS Lett 587**,** 3182-3187.

34. Vazquez, M. P. and Levin, M. J., 1999. Functional analysis of the intergenic regions of TcP2beta gene loci allowed the construction of an improved Trypanosoma cruzi expression vector. Gene 239**,** 217-225.
35. Veron, M., Tepper, A., Hildebrandt, M., Lascu, I., Lacombe, M. L., Janin, J., Morera, S., Cherfils, J., Dumas, C., and Chiadmi, M., 1994. Nucleoside diphosphate kinase: an old enzyme with new functions? Adv Exp Med Biol 370**,** 607-611.
36. von Stetten, D., Noirclerc-Savoye, M., Goedhart, J., Gadella, T. W., Jr., and Royant, A., 2012. Structure of a fluorescent protein from Aequorea victoria bearing the obligate-monomer mutation A206K. Acta Crystallogr Sect F Struct Biol Cryst Commun 68**,** 878-882.
37. Yoshida, Y. and Hasunuma, K., 2006. Light-dependent subcellular localization of nucleoside diphosphate kinase-1 in Neurospora crassa. FEMS Microbiol Lett 261**,** 64-68.
38. Zhang, S. Q., Hu, Y., and Jong, A. Y., 1995. Temporal and spatial distributions of yeast nucleoside diphosphate kinase activities and its association with the Cdc8p. Cell Mol Biol Res 41**,** 333-346.
39. Zingales, B., Andrade, S. G., Briones, M. R., Campbell, D. A., Chiari, E., Fernandes, O., Guhl, F., Lages-Silva, E., Macedo, A. M., Machado, C. R., Miles, M. A., Romanha, A. J., Sturm, N. R., Tibayrenc, M., and Schijman, A. G., 2009. A new consensus for Trypanosoma cruzi intraspecific nomenclature: second revision meeting recommends TcI to TcVI. Mem Inst Oswaldo Cruz 104**,** 1051-1054.

Figure captions

Figure 1: *Subcelullar localization of N1*. **A.** Immunofluorescences carried out over wild type (WT) and N1 overexpressing epimastigotes (N1). The parasites were settled onto poly-L-lysine coated coverslips, fixed, permeabilized and incubated with specific mouse anti-N1 serum and DyLight 488- conjugated anti-mouse. Scale bar: 5 µm. **B.** Digitonin extraction of N1. Supernatant and pellet fractions were analyzed by Western Blot using anti-N1 or anti-GDH serum ($NADP^+$ -linked glutamate dehydrogenase) as cytosolic marker.

Figure 2: *Protein expression analysis of wild type and transgenic parasites*. **A.** Western Blot from SDS-PAGE. N1 overexpressing parasites (1) and wild type parasites (2), were checked using anti-N1 serum; N1-HE (3) and N1-Ch (4) overexpressing parasites were analyzed with anti-HA antibodies; GFP (5), N1P95S-G (6), N1-G (7), N1-mG (8) and N1H117N-G (9) overexpressing epimastigotes were examined using anti-GFP antibodies. **B.** Western Blot from native PAGE. GFP (1), N1P95S-G (2), N1-G (3), N1-mG (4) and N1H117N-G (5) overexpressing epimastigotes were examined using anti-GFP antibodies. Arrows indicate different oligomeric structures, note that N1P95S-G has more mobility than the other constructs. **C**. Western Blot using anti-N1 serum of N1-G (1), N1-mG (2), N1P95S-G (3) and N1H117N-G (4) transgenic parasites.

Figure 3: *N1 granules*. **A.** Confocal microscopy of GFP and N1-G overexpressing parasites. The cells were settled onto poly-L-lysine coated coverslips, fixed and analyzed for direct fluorescence. Arrows indicate the observation of granules under phase contrast microscopy. **B.** Transmission electron microscopy of GFP and N1-G overexpressing parasites. a-b-d-e) N1-G cells, b) zoom of image a, c) GFP overexpressing parasites. a-c-d-e) 8,600x b) 35,000x. N: nucleus, K: kinetoplasto, F: flagellum, G: N1-G granule. Arrows indicate the N1-G granules.

Figure 4: *Analysis of granule formation*. Fluorescence microscopy of parasites overexpressing wild type or mutated N1 fused with different tags. **A.** N1-HE: N1 fused with the low molecular weight epitopes HA-EEF; N1-Ch: N1 fused with the monomeric fluorescent protein mCherry; N1-mG: N1 fused with monomeric A206K GFP; N1P95S-G: N1 bearing a mutation in Pro95 and in the C-terminus that destabilize the hexamer fused with GFP; N1H117N-G: Inactive N1 mutated in His117 fused with GFP; GDH-G: $NADP^+$-linked glutamate dehydrogenase fused with GFP. **B.** N1-G – N1-Ch: Co-expression of N1 fused with GFP and with mCherry. In both figures the parasites were analyzed by direct fluorescence. In the particular case of N1-HE the construct was observed by immunofluorescence with anti-HA antibodies. Scale bar: 5 µm.

Figure 5: *Analysis of granules in other stages of the parasite and under stress conditions.* Different stages of parasites overexpressing N1-HE fusing protein were analyzed by immunofluorescence using anti-HA antibodies. A) Trypomastigotes obtained from infected cells; B) metacyclic trypomastigotes; C) intracellular amastigotes; D) metacyclic tryposmastigotes; E) epimastigotes in stationary phase of culture; F) and G) epimastigotes in TAU-P medium. Arrows indicate the presence of large granules. Scale bar: 5 µm.

Figure 1

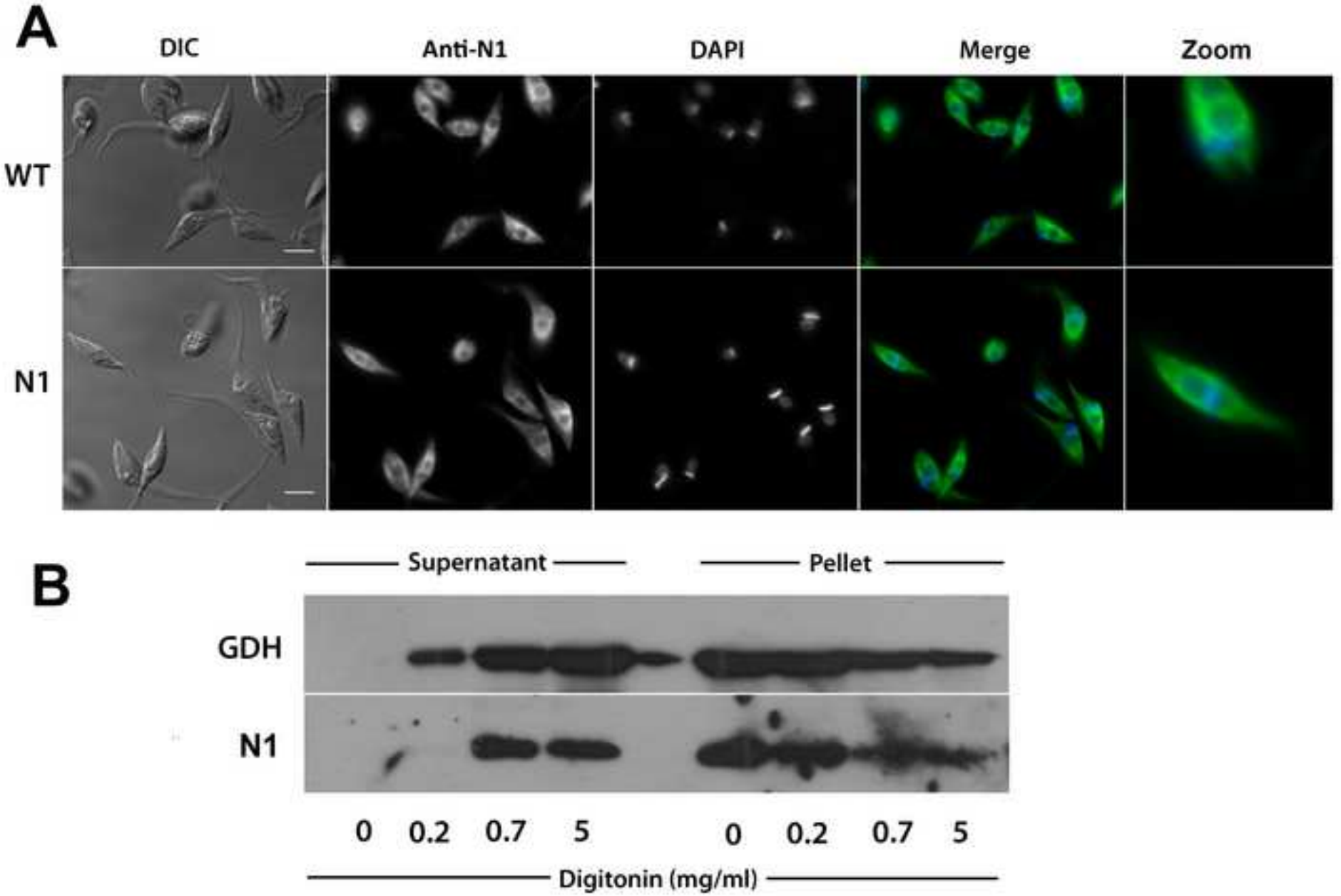


Figure 2

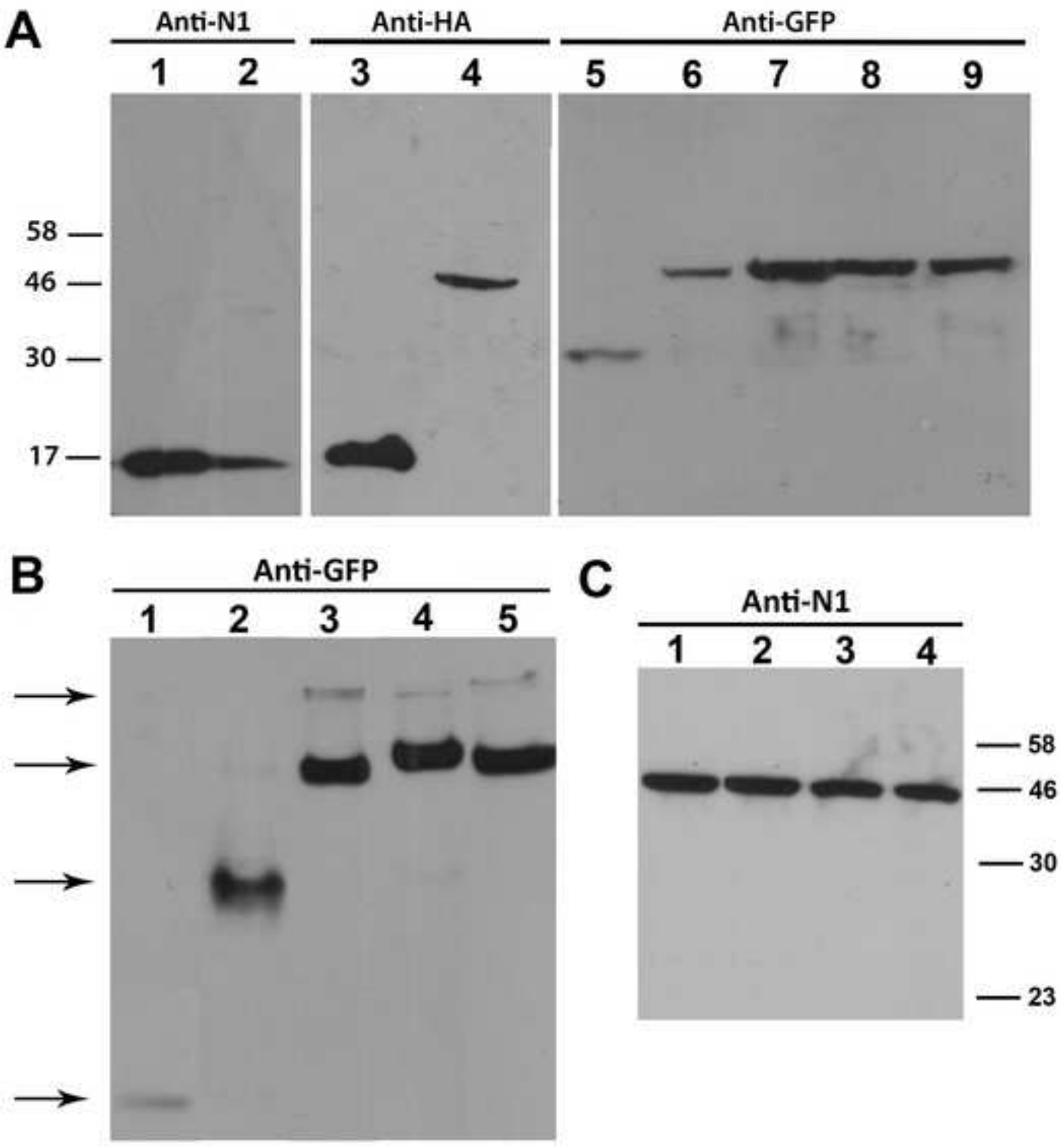


Figure 3

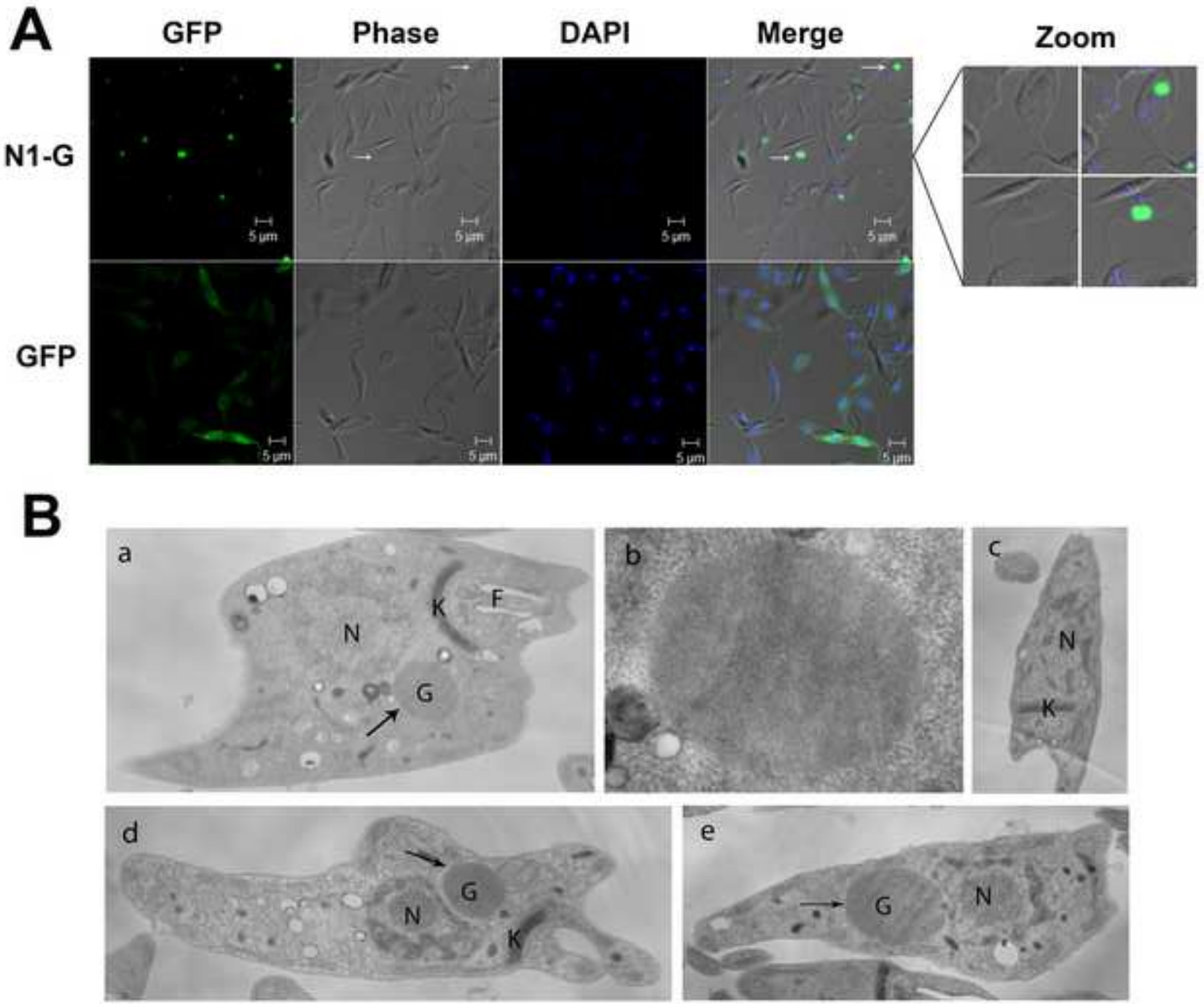


Figure 4

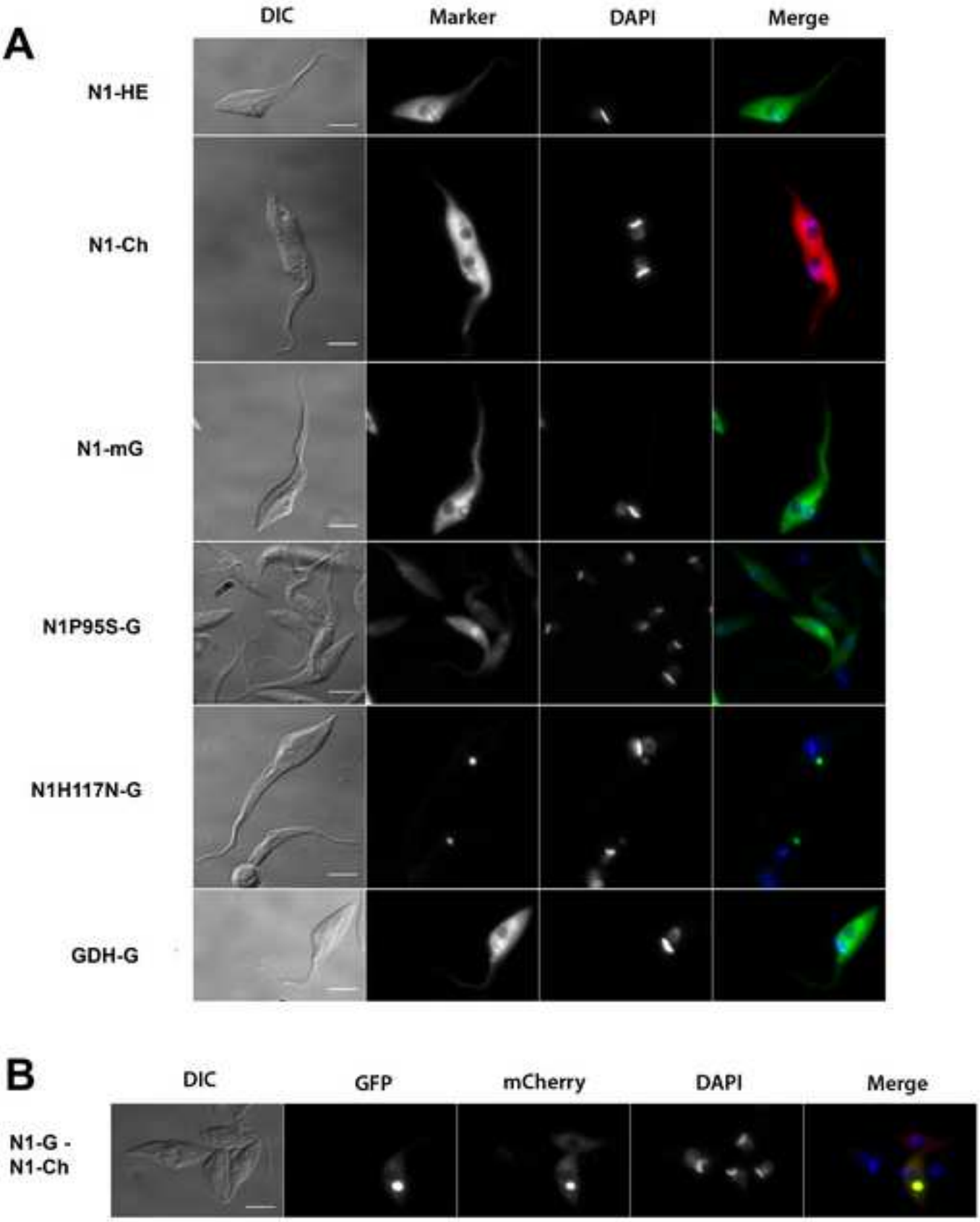
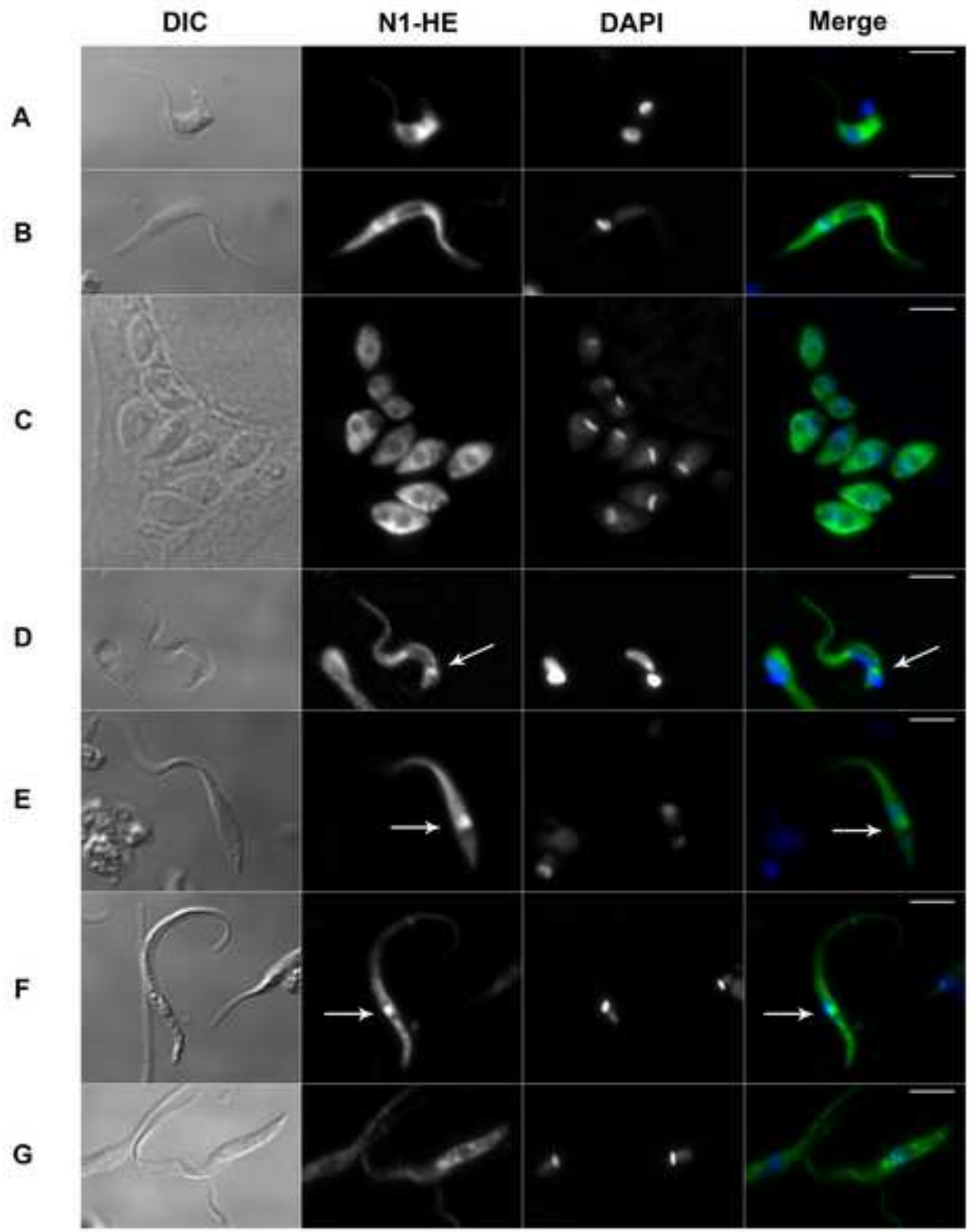
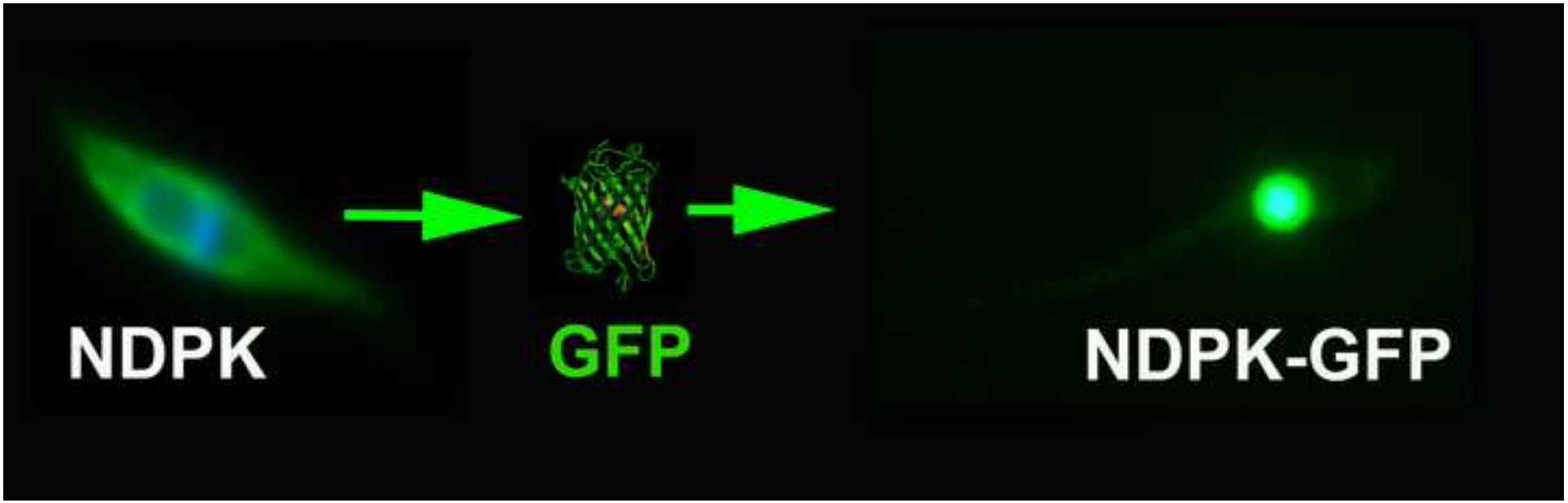


Figure 5



Highlights

- Nucleoside diphosphate kinase 1 (NDPK1) from *Trypanosoma cruzi* has a cytosolic localization
- NDPK1 generates large membrane-free granules that depend on its hexameric structure
- Granules are specific for the canonical isoform of NDPK
- Granules are present in metacyclic trypomastigotes and stressed epimastigotes


NDPK
GFP
NDPK-GFP