Investigating DNA Damage responses in Apicomplexan parasites

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- Across the Apicomplexan phylum, DNA damage response pathway(s) are not well characterised.
- Some Apicomplexans have retained homologs of phosphatidylinositol 3-kinase-related kinases (PIKKs) such as ATM and ATR, which signal cell cycle checkpoints, whereas others, including *Plasmodium*, have not. In *Toxoplasma gondii,* there are putative homologs of ATM and ATR.
- Here we investigated the role of one of the *T. gondii* PIKKs, '*Tg*ATM' (TGGT1_248530).
- **We report that *Tg*ATM is necessary for the phosphorylation of a DNA damage marker, but is non-essential in cultured tachyzoites, and that the action of the Human ATM inhibitor KU-55933(KU) in *T. gondii* is more complex than originally thought.**

Human PIKK homologs present in *T.gondii*

Human PIKK homologs are present in *T.gondii* (fig 1A). Both were associated with reduced fitness in a genome-wide loss-of-function CRISPR screen[1].

A

Gene ID (e.g., TGME49)	NLS	FAT	PRD	FATC	MW (kDa)	Blastp	Phenotype Score[a]
_248530	2	NO	ND	1	246	HuATM	−2.71
_266010	1	NO	ND	1	964	DNA-PK	−3.15
_268370	2	1	1	1	904	HuTRRAP	−3.56
_283702	NO	NO	1	1	647	HuATR	−2.68
_316430	2	1	1	1	543	mTOR	0.21

These PI3,4K putative proteins were retrieved from www.toxodb.org based on Blast analysis by using Human ATM (AAB65827), ATR (CAA70298), and DNA-PK (AAB39925) aminoacidic sequences. Domains and motifs were searched by using motifscan (http://hits.isb-sib.ch/cgi-bin/PFSCAN). FAT, FRAP (FKBP12-rapamycin-associated protein)-ATM-TRRAP (Transformation/transcription domain-associated protein) domain; PRD, PIKK regulatory domain; FATC, FAT-C-terminal domain. HuTRRAP, transformation/transcription domain-associated protein, isoform CRA_e (EAW76697). mTOR: mammalian Target Of Rapamycin (NP_004949).

ND: not detected

[a] "Genome-wide loss of function screen (CRISPR) that measures each gene's contribution to Toxoplasma gondii fitness during infection of human fibroblasts. Phenotype score = log2 (sgRNA of infected cultures/sgRNA composition of original library)" (www.toxodb.org). Negative score, fitness conferring; positive score, dispensable.

B

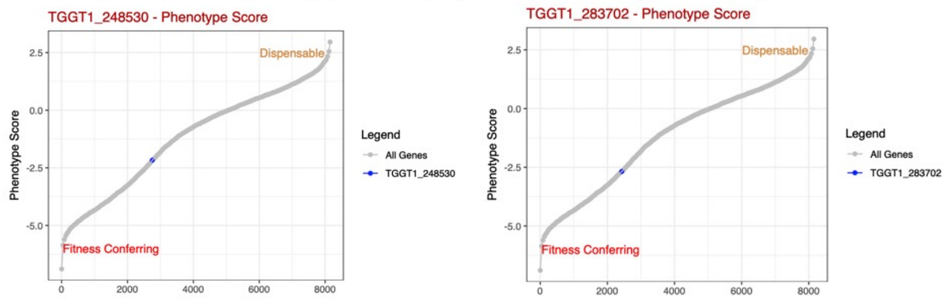


Figure 1: A. Genes in *T.gondii* found by blast analysis of their human homologs. All PIKKs contain a FATC domain; presence/absence of other domains is annotated. Phenotype scores from a CRISPR fitness screen also shown. **B.** fitness curves for *T.gondii* genes in the CRISPR screen[1].

*Tg*ATM is not essential

An inducible knockdown of *Tg*ATM was made using a Tet OFF system with 24h addition of ATC (fig 2B). *Tg*ATM was shown to be non-essential (fig 2A). Knockdown of *Tg*ATM did not sensitize parasites to DNA damage (MMS) (fig 2A,C). Under CPT treatment cells behave in a similar way to MMS treatment.

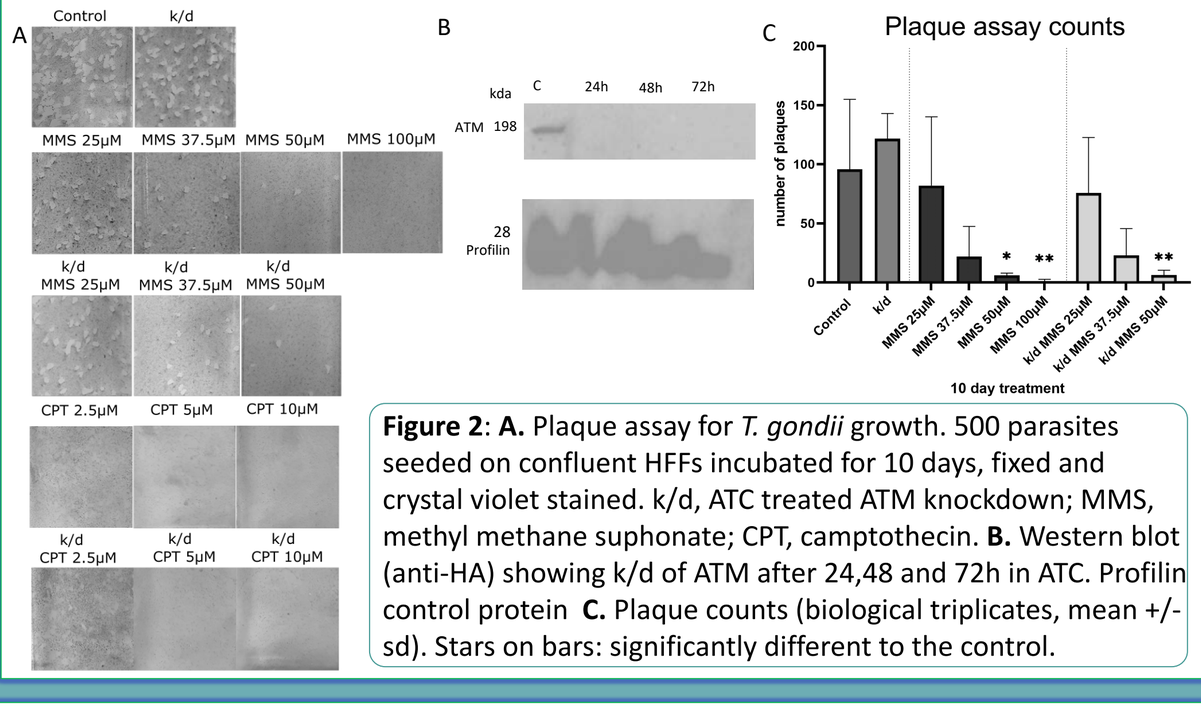


Figure 2: **A.** Plaque assay for *T. gondii* growth. 500 parasites seeded on confluent HFFs incubated for 10 days, fixed and crystal violet stained. k/d, ATC treated ATM knockdown; MMS, methyl methane suphonate; CPT, camptothecin. **B.** Western blot (anti-HA) showing k/d of ATM after 24,48 and 72h in ATC. Profilin control protein **C.** Plaque counts (biological triplicates, mean +/- sd). Stars on bars: significantly different to the control.

A human ATM inhibitor may have off-target effects in *T.gondii.*

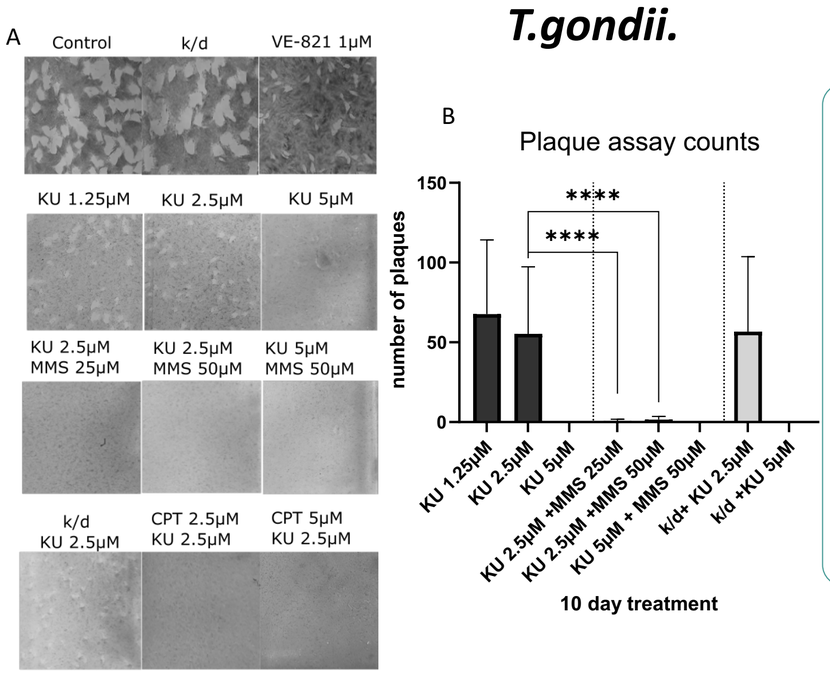
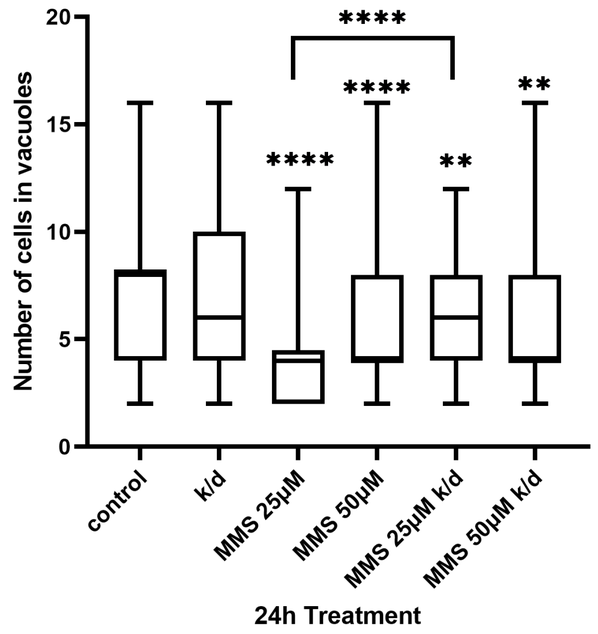


Figure 3: A. Plaque assay for *T. gondii* growth. 500 parasites seeded on confluent HFFFs incubated for 10 days, fixed and crystal violet stained. MMS, methyl methane suphonate; KU, ATM inhibitor KU-55933; VE-821, ATR inhibitor VE-821; CPT, camptothecin **B.** Plaque counts (biological triplicates, mean +/- sd).

Drugs developed to inhibit human ATM (KU) or ATR (VE-821) both affect *T.gondii* growth, whereas ATM k/d does not. (Addition of KU plus CPT or MMS kills parasites.) Further exploration of these drugs' effects is required, along with a full investigation of the role of *Tg*ATR.

Knockdown of *Tg*ATM can reduce the impact of DNA damage on replication progression



In model eukaryotes ATM is involved in signaling the initiation of checkpoints to repair DNA after damage[2,3]. This could slow the progression of replication in DNA damaged cells. A replication assay in *Tg*ATM k/d parasites showed that they replicate more after low-level DNA damage than the parental line, suggesting impaired checkpoint activity (fig 4).

Figure 4: Box and whisker plot of replication assay. Parasites seeded on confluent HFFFs, allowed 2h for invasion, then treated for 24h with drug treatments. ATC treated cells were treated for 24h before seeding again, to ensure full knockdown of *Tg*ATM. Cells were fixed and the number of Toxoplasma counted for 250 vacuoles. Stars on bars: significantly different to control, at 25 μM MMS control replication differs significantly from k/d.

Knockdown of *Tg*ATM prevents phosphorylation of a DNA damage marker

In humans and some Apicomplexan parasites the phosphorylation of a histone H2AX has been shown to be a marker of DNA damage[4]. In humans this is completed by ATM[4]. A previous publication showed that the KU-55933 inhibitor prevented H2AX phosphorylation in *T. gondii* after DNA damage, presumptively via inhibition of *Tg*ATM[5].

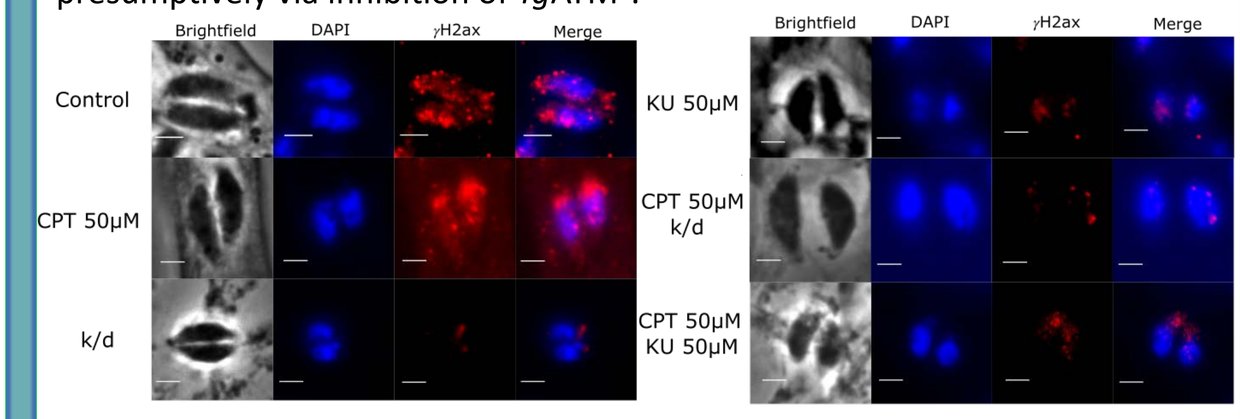


Figure 5: Immunofluorescence assay using phosphorylated Histone H2AX human antibody. Parasites seeded on confluent HFFs incubated for 24h (ATC added to k/d), treatment added for 2h, CPT 20xIC50, KU 20xIC50. Cells fixed and stained with anti-γH2AX (red) and DAPI (blue). Scale bar 2μm. KU, ATM inhibitor KU-55933; CPT, camptothecin double strand break agent.

Fig 5 shows that the knockdown of *Tg*ATM does inhibit H2AX phosphorylation, as does KU. However KU has a greater effect than the knockdown, suggesting other targets than ATM.

Conclusions and future considerations

- *Tg*ATM is not essential in cultured tachyzoites, although it does appear to have some effect on replication after DNA damage (checkpoint signalling).
- Results call into question the specificity of a human ATM inhibitor in *T. gondii.*
- There is a putative ATR in *T.gondii* which may also be affected by the inhibitors: this protein will also be knocked down and activity explored.
- Using this system, complementation will be attempted with PI3K, the closest PIKK homolog left in *Plasmodium*, to add more to the picture of DNA damage repair mechanisms in Apicomplexans.

References

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