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DISCOVERIES FOR HUMANITY

# Genetic regulation and innate stress responses in metronidazole resistance in *Giardia*


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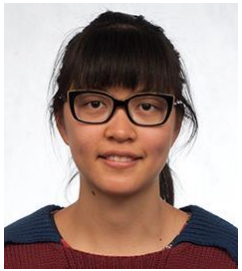
Brendan


Louise


Nijoy


Sam


Joy


Katharina


Balu

CANCER | IMMUNE DISORDERS | INFECTIOUS DISEASE

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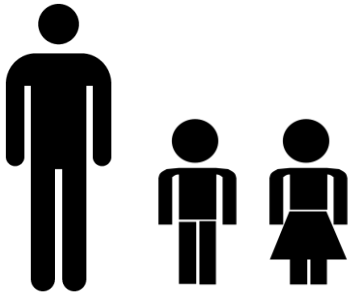
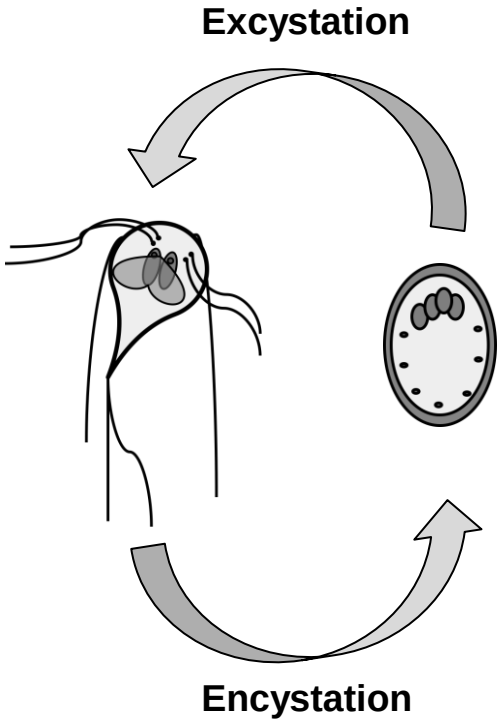
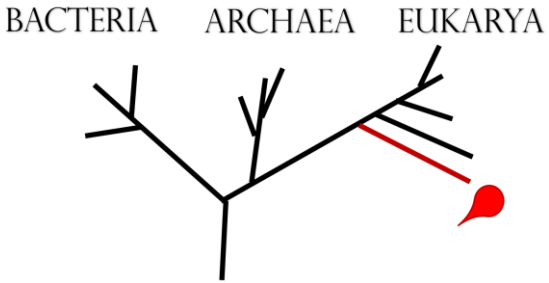
# *Giardia duodenalis* and Giardiasis



- 1 billion infected
- 200-300 million cases
- Children <5 years
- Post-infectious sequelae




- Compact genome
- Amitochondriate
- Bacterial metabolism
- Reduced eukaryote cell biology

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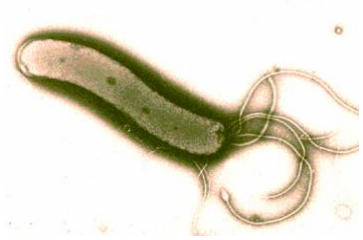
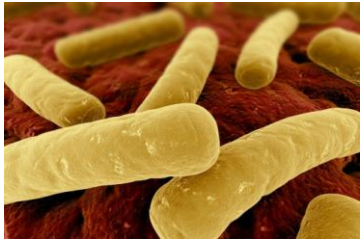
# Metronidazole – anaerobic/microaerophilic pathogens

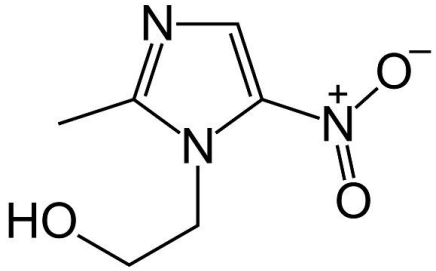


*Helicobacter pylori*

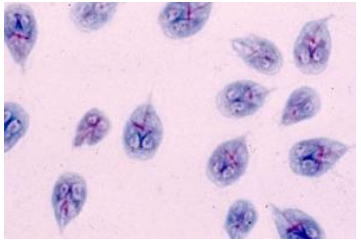
*Bacteriodes fragilis*



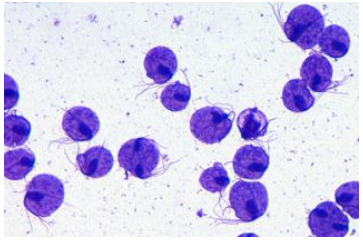
*Clostridium sp.*



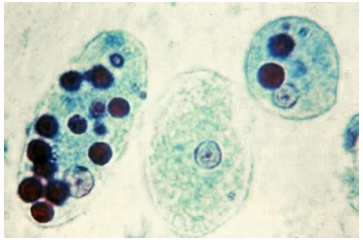

- Nitroheterocyclic
- Pro-drug
- Specific for low dissolved oxygen (reduced)
- Oxidative damage to biomolecules



*Giardia duodenalis*



*Trichomonas vaginalis*



*Entamoeba histolytica*

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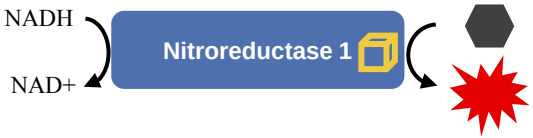
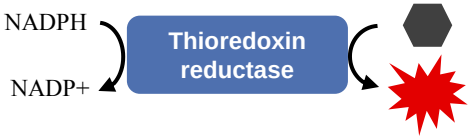
# Metronidazole – activation and detoxification

## ACTIVATION

Toxic intermediates

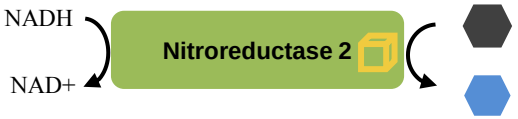


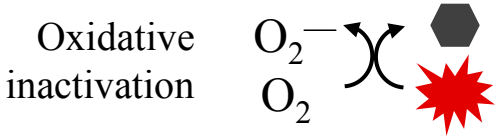





## DETOXIFICATION

Inert amine





Townson et al., *Mol Biochem Parasitol* 1996
Nillius et al., *Antimico Chemother* 2011
Leitsch et al., *Antimico Chemother* 2011
Müller et al., *Antimico Chemother* 2013
Leitsch et al., *Int J Parasitol* 2016

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# Resistance phenotypes – incomplete & complex

| Line | Selected resistance | Cross-resistance | Infectivity | MET activation: PFOR (PFOR-1 17063) | MET activation: PFOR (PFOR-2 114609) | MET activation: Fd (Fd-1 9662) | MET activation: TrxR (9827) | MET activation: NR-1 (22677) | MET detoxification: NR-2 (6175) | NADPH metabolism: NADPH oxidase (†) | NADPH metabolism: GDH (21942) | Protein chaperones & HDACs: PDI-2 (9413) | PDI-4 (103713) | Sir-2 (16569) | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WB-M3 | MET (UV) | | | a ↓ | | | | | | | | | | | Townson et al. (1996) |
| 713-M3 | MET (UV) | • | 0 | a ↓ | | a ↓ | | t ↓* | t ↑* | a ↓ | | | | | Leitsch et al. (2011), Müller et al. (2013), Liu et al. (2000), Townson et al. (1996) |
| WB-C5 | MET | | | t ↓ | t ↓ | | | t - | t - | | t ↓ | t ↑ | t - | | Müller et al. (2007a), Müller et al. (2013) |
| 1062ID$_{10}$ | MET | TIN, ORN | $10^5$ | a ↓ | | a - | a ↑ | t - | t ↑* | a ↓ | | | | | Leitsch et al. (2011), Smith et al. (1988), Müller et al. (2013), Townson et al. (1996) |
| WB-M1 | MET (UV) | • | 0 | | t ↓* | t ↓* | t - | t ↓* | | | | | | | Tejman-Yarden et al. (2011) |
| WB-M2 | MET (UV) | TIN, ORN | $10^6$ | | t - | t ↓* | t - | t ↓* | | | | | | | Tejman-Yarden et al. (2011) |
| 713-M3-C17 | MET (UV), C17 | TIN, ORN | | a ↑ | | a - | | t ↓* | t - | a ↓ | | | | | Dunn et al. (2010), Leitsch et al. (2011), Müller et al. (2013) |
| 106-C17 | C17 | MET | 0 | a - | | a - | | t ↓* | t - | a ↓ | | | | | Dunn et al. (2010), Leitsch et al. (2011), Müller et al. (2013) |
| WB-C4 | NTZ | MET | | t ↑* | t - | t - | t - | t ↓* | t - | t - | t ↓ | t ↑* | t ↑* | t ↓* | Müller et al. (2007a, 2008, 2013), Nillius et al. (2011) |
| NTZII | NTZ | | | | | | | t ↓* | | | | | | | Nillius et al. (2011) |

PFOR Fd TrxR NR1 NR2

ACTIVATION DETOXIFICATION

- What underpins Mtz resistance?
- How does response to Mtz differ from other stress responses?
- What regulates these responses?
- Are there multiple pathways to Mtz resistance?
- Are these pathways equal in clinical relevance?

Ansell et al., *Biotech Adv* 2015

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# What happens in *Giardia* culture?

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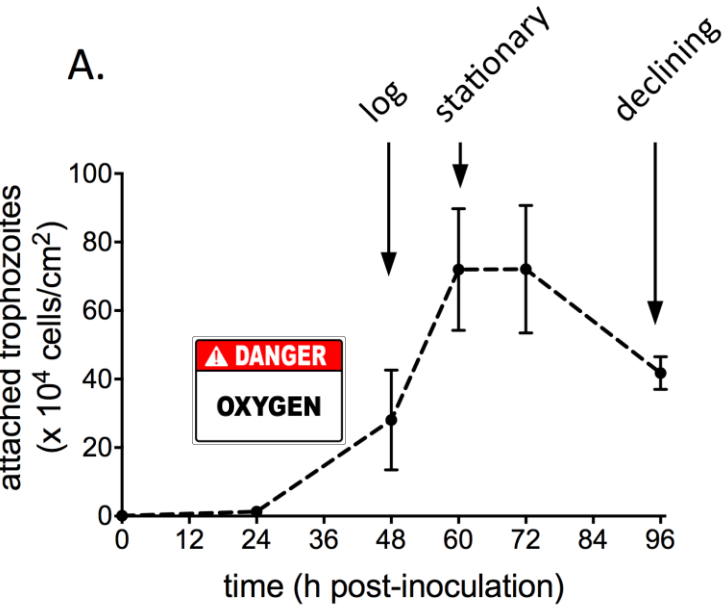
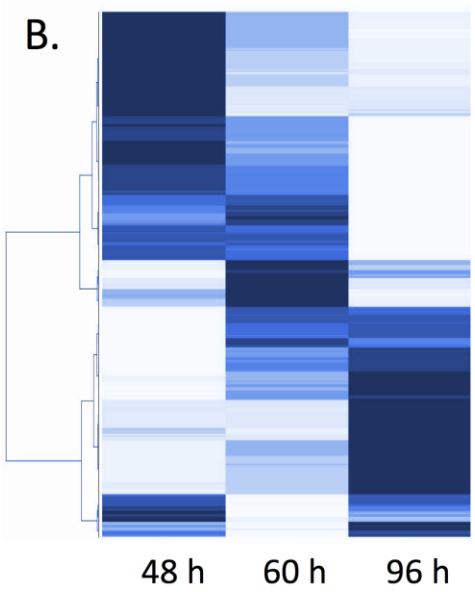
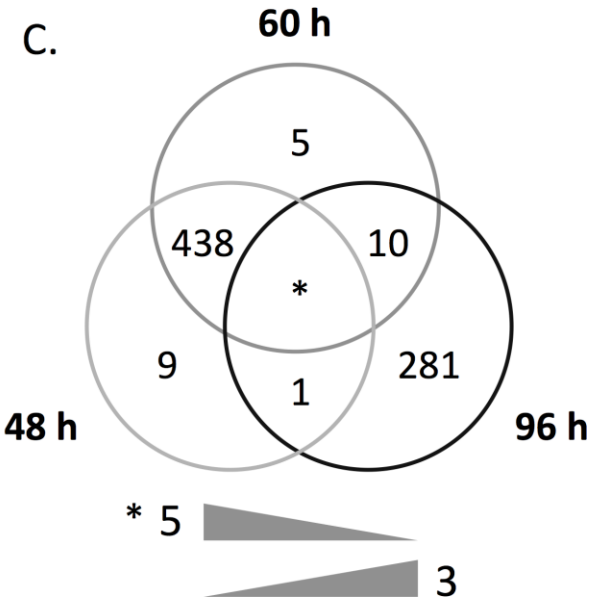
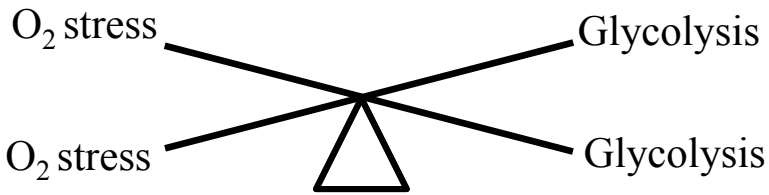
# Growth, metabolism and oxidative stress



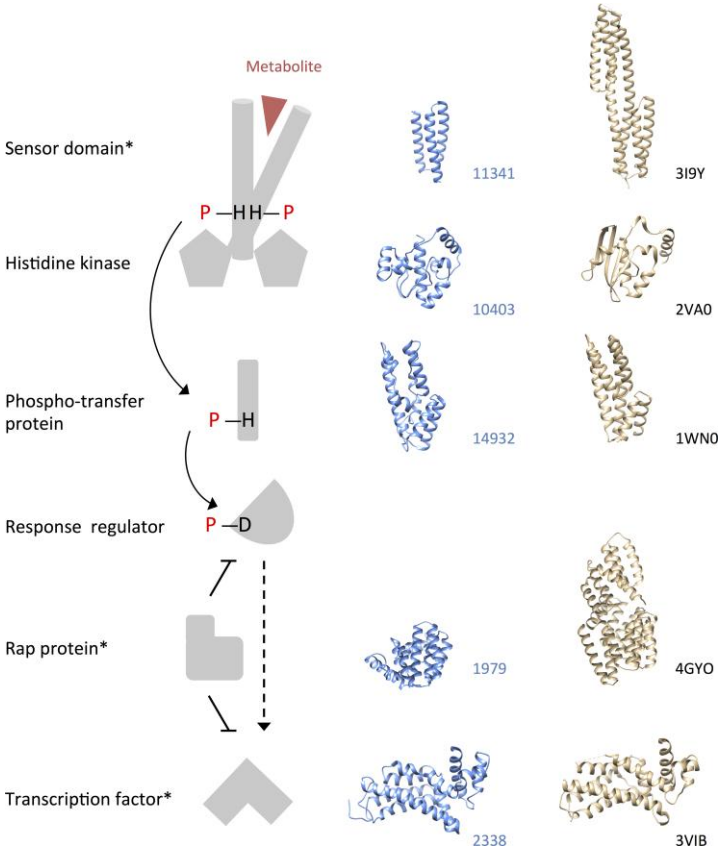



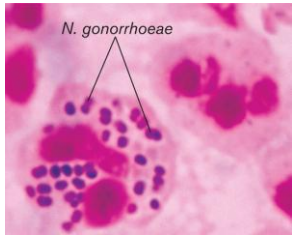



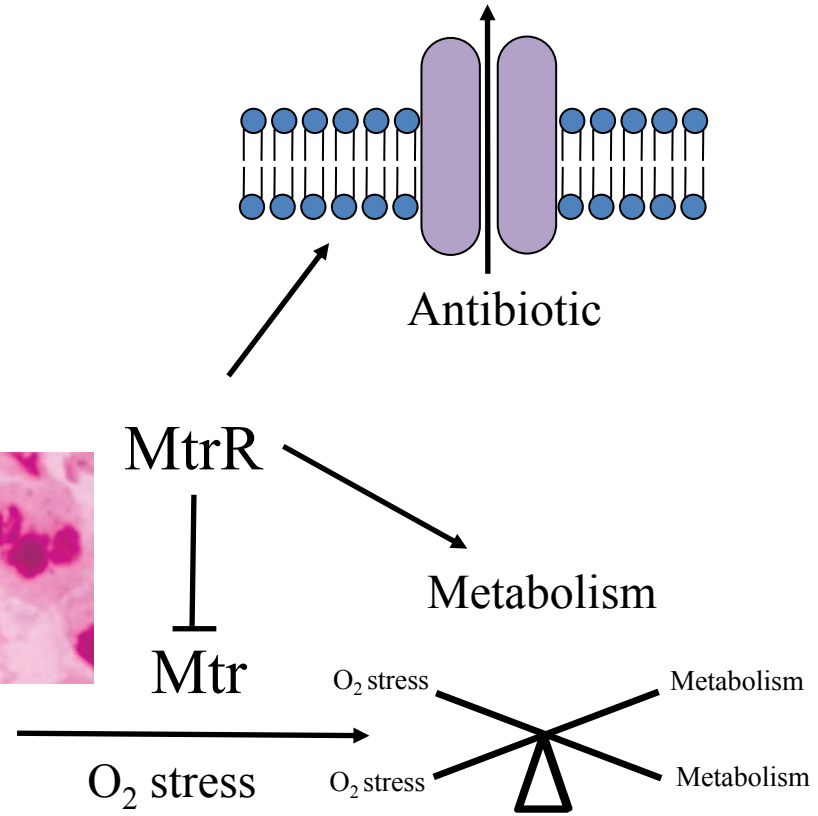




Ansell et al., *PLoS NTD* 2016

# Stress response regulation vs drug resistance?




Ansell et al., *PLoS NTD* 2016

Folster et al, *J Bacterol,* 2009; Johnson and Shafer, *Antibiotics*, 2015

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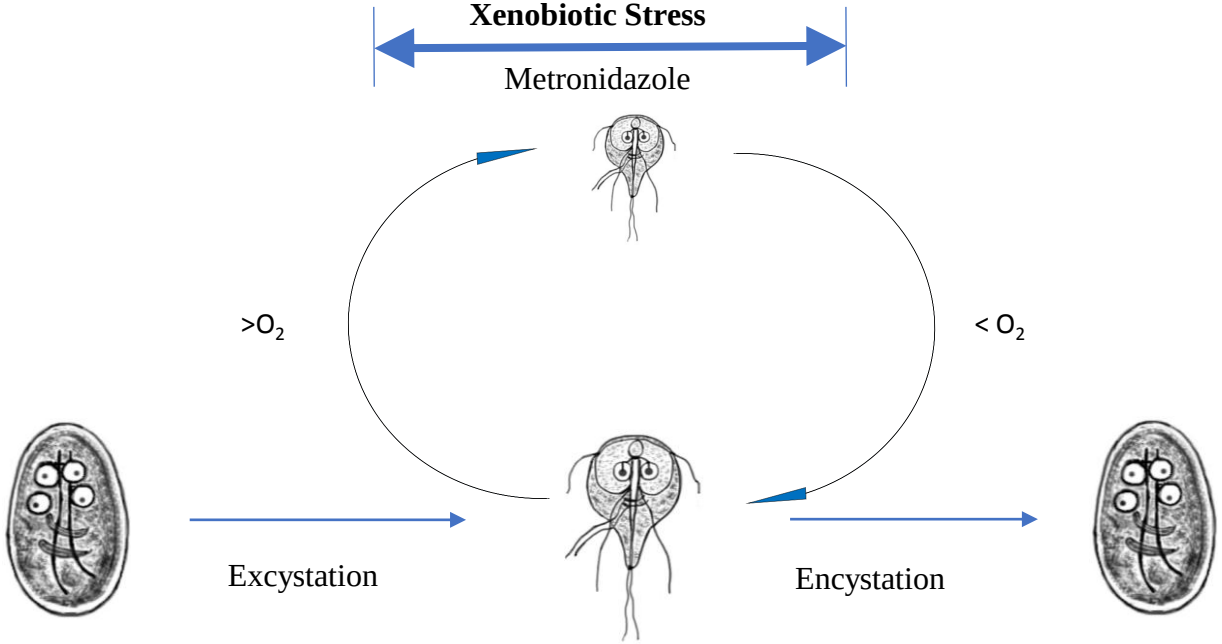
# How do drug and stress responses compare?

# *Giardia* mounts specific stress responses




(Spycher et al;2013 B. Ansell et al; 2015, B. Ansell et al; 2016, B. Ansell et al; 2017)

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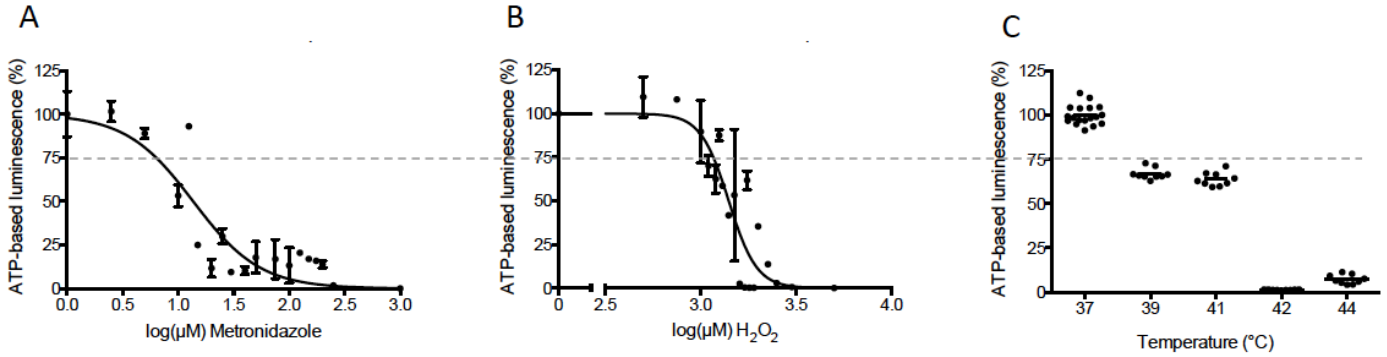
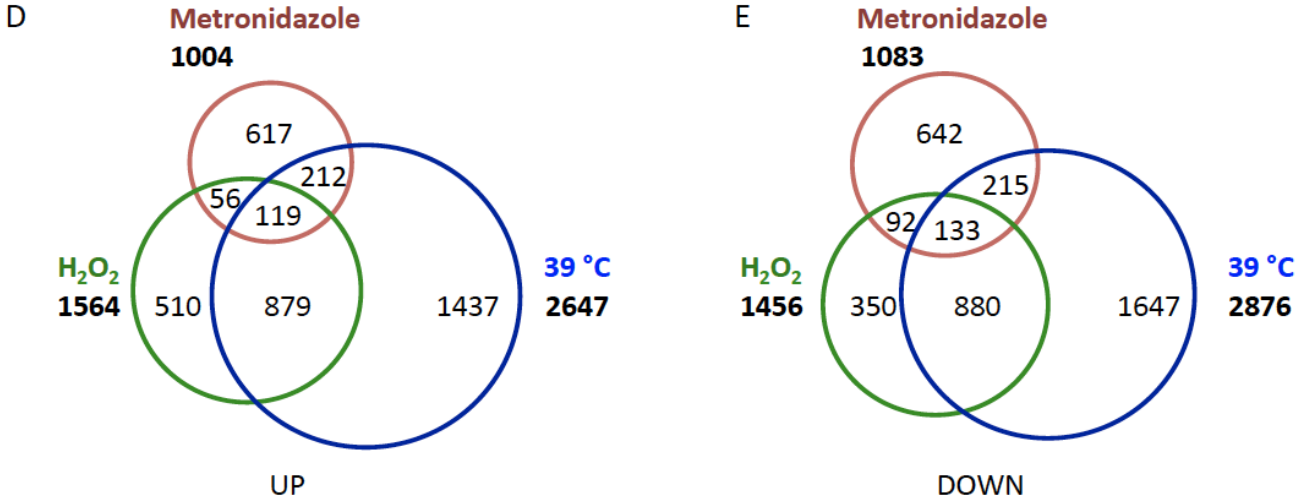
# *Giardia* mounts stress specific responses




Ansell et al., *AAC* 2016

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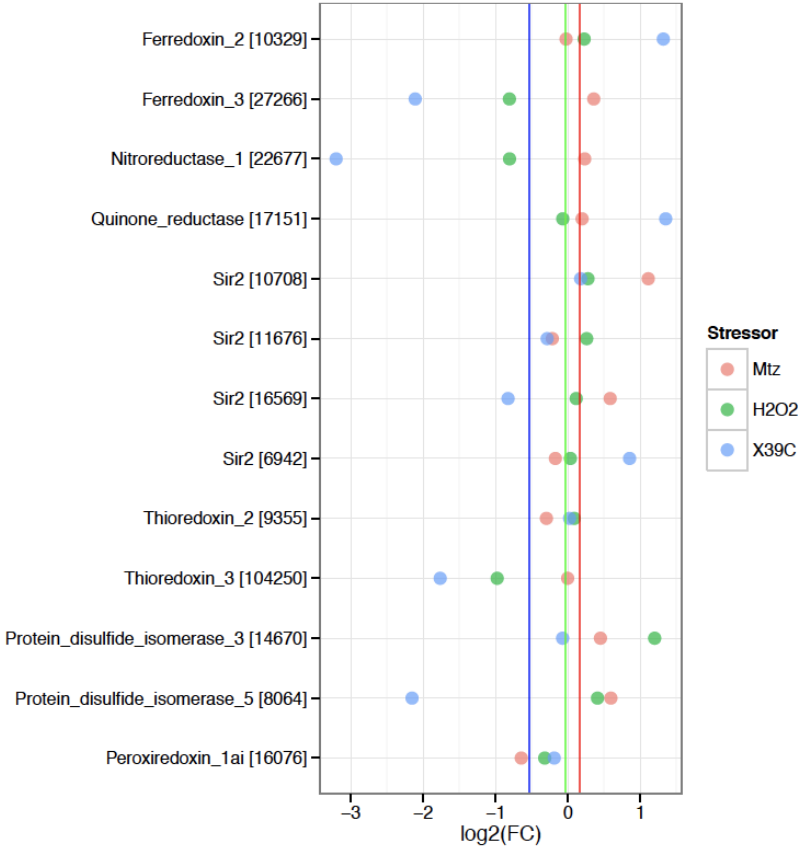
# *Giardia* mounts stress specific responses


Ansell et al., *AAC* 2016

| Line | Selected resistance | Cross-resistance | Infectivity | MET activation: PFOR (PFOR-1 17063) | MET activation: PFOR (PFOR-2 114609) | MET activation: Fd (Fd-1 9662) | MET activation: TrxR (9827) | MET activation: NR-1 (22677) | MET detoxification: NR-2 (6175) | NADPH metabolism: NADPH oxidase (†) | NADPH metabolism: GDH (21942) | Protein chaperones & HDACs: PDI-2 (9413) | Protein chaperones & HDACs: PDI-4 (103713) | Protein chaperones & HDACs: Sir-2 (16569) | References |
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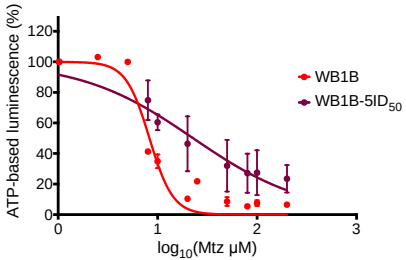
| WB-M3 | MET (UV) | | | a ↓ | | | | | | | | | | | Townson et al. (1996) |
| 713-M3 | MET (UV) | • | 0 | a ↓ | | a ↓ | | t ↓* | t ↑* | a ↓ | | | | | Leitsch et al. (2011), Müller et al. (2013), Liu et al. (2000), Townson et al. (1996) |
| WB-C5 | MET | | | t ↓ | t ↓ | | | t - | t - | | t ↓ | t ↑ | t - | | Müller et al. (2007a), Müller et al. (2013) |
| 1062ID$_{10}$ | MET | TIN, ORN | $10^5$ | a ↓ | | a - | a ↑ | t - | t ↑* | a ↓ | | | | | Leitsch et al. (2011), Smith et al. (1988), Müller et al. (2013), Townson et al. (1996) |
| WB-M1 | MET (UV) | • | 0 | | t ↓* | t ↓* | t - | t ↓* | | | | | | | Tejman-Yarden et al. (2011) |
| WB-M2 | MET (UV) | TIN, ORN | $10^6$ | | t - | t ↓* | t - | t ↓* | | | | | | | Tejman-Yarden et al. (2011) |
| 713-M3-C17 | MET (UV), C17 | TIN, ORN | | a ↑ | | a - | | t ↓* | t - | a ↓ | | | | | Dunn et al. (2010), Leitsch et al. (2011), Müller et al. (2013) |
| 106-C17 | C17 | MET | 0 | a - | | a - | | t ↓* | t - | a ↓ | | | | | Dunn et al. (2010), Leitsch et al. (2011), Müller et al. (2013) |
| WB-C4 | NTZ | MET | | t ↑* | t - | t - | t - | t ↓* | t - | t - | t ↓ | t ↑* | t ↑* | t ↓* | Müller et al. (2007a, 2008, 2013), Nillius et al. (2011) |
| NTZII | NTZ | | | | | | | t ↓* | | | | | | | Nillius et al. (2011) |

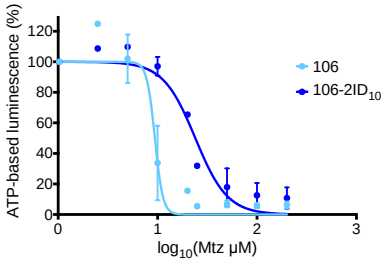
PFOR Fd TrxR NR1 NR2

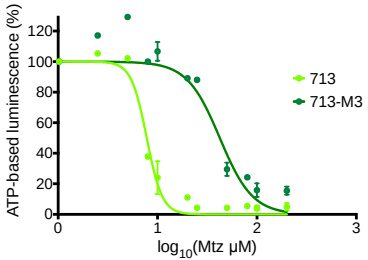
ACTIVATION DETOXIFICATION

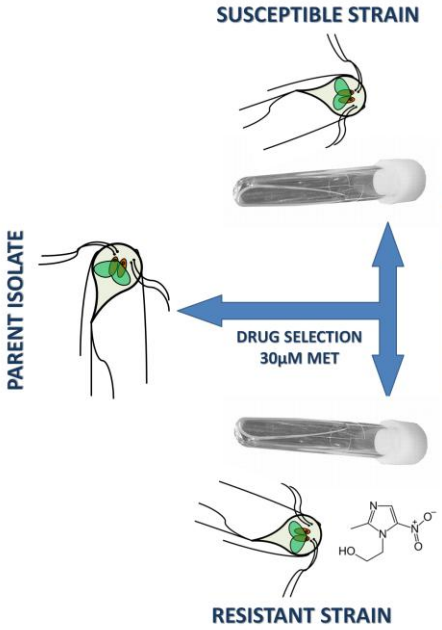
# Are all *Giardia* Mtz responses equal?

# 3-way isogenic isolate (Mtz-R vs Mtz-S) analysis



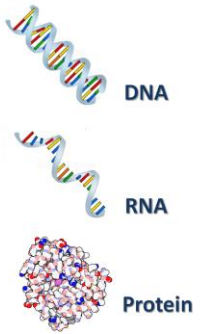




TRIPURE (ROCHE) WHOLE TROPHOZOITE EXTRACTION

IC50 DETERMINATION-- CELLTITRE-GLO ATP-BASED LUMINESCENCE ASSAY

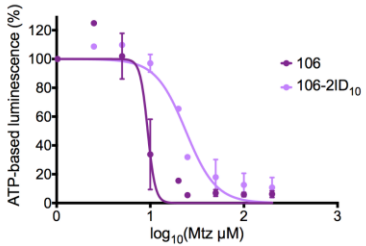
| Isolate | Sus IC50 | Res IC50 | Resistance Factor (RF) |
|---|---|---|---|
| WB | 8.28 µM | 22.79 µM | 2.8 |
| 106 | 9.39 µM | 23.99 µM | 2.6 |
| 713 | 7.79 µM | 42.33 µM | 5.4 |

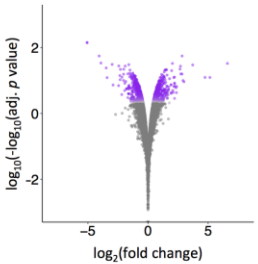


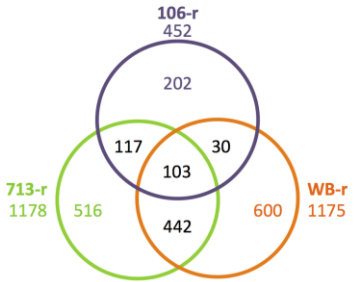

TRIPURE (ROCHE) WHOLE TROPHOZOITE EXTRACTION

# Resistant *vs* susceptible — differential transcription

106-r

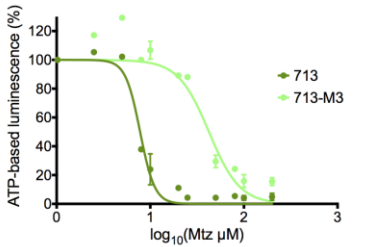


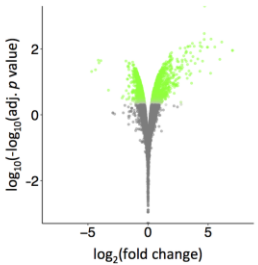



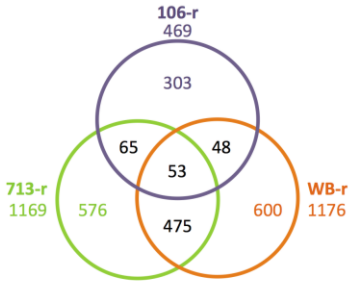




Increased transcription

713-r

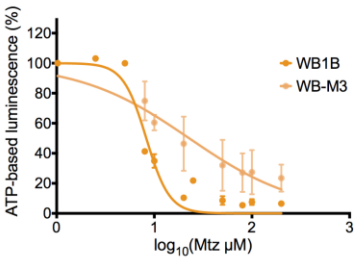


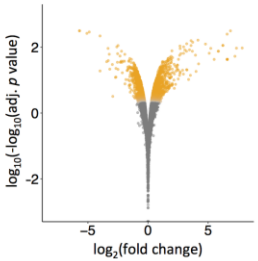




Decreased transcription

WB-r

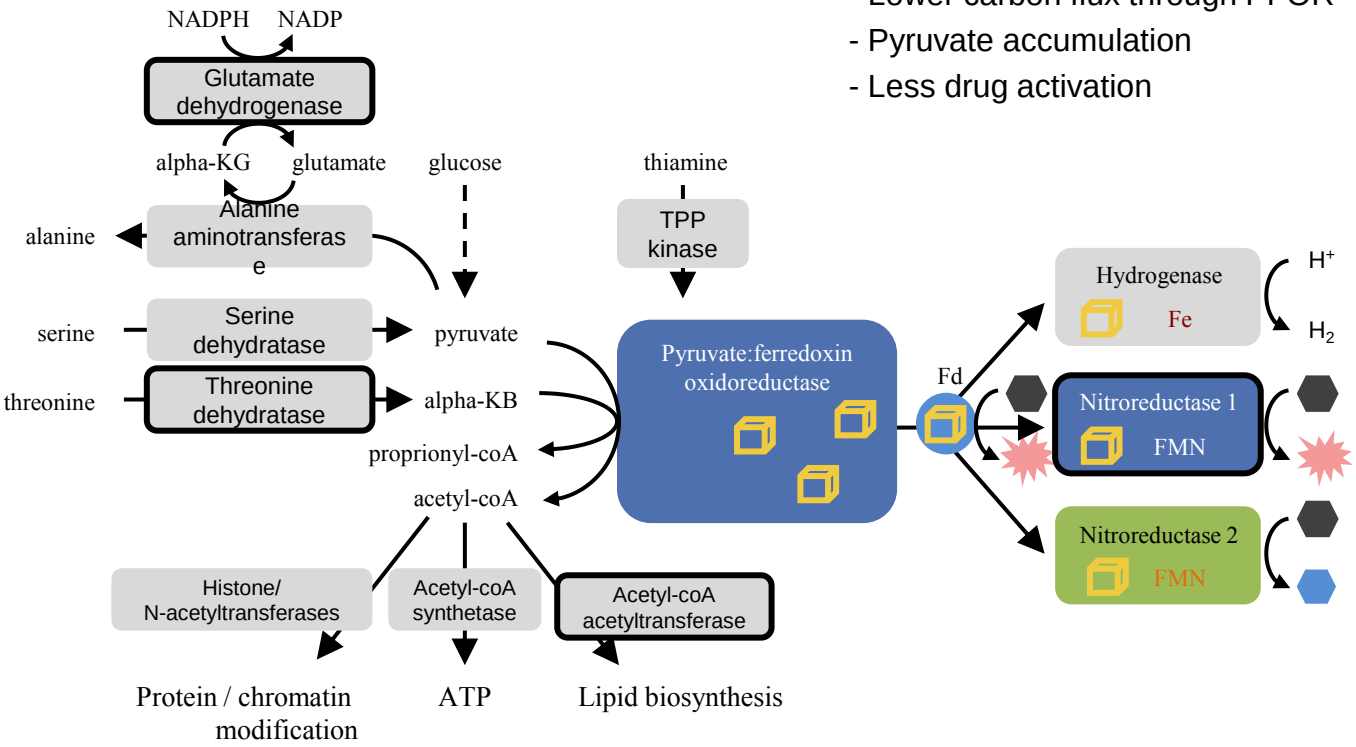



Ansell et al., *Frontiers Microbiol* 2017

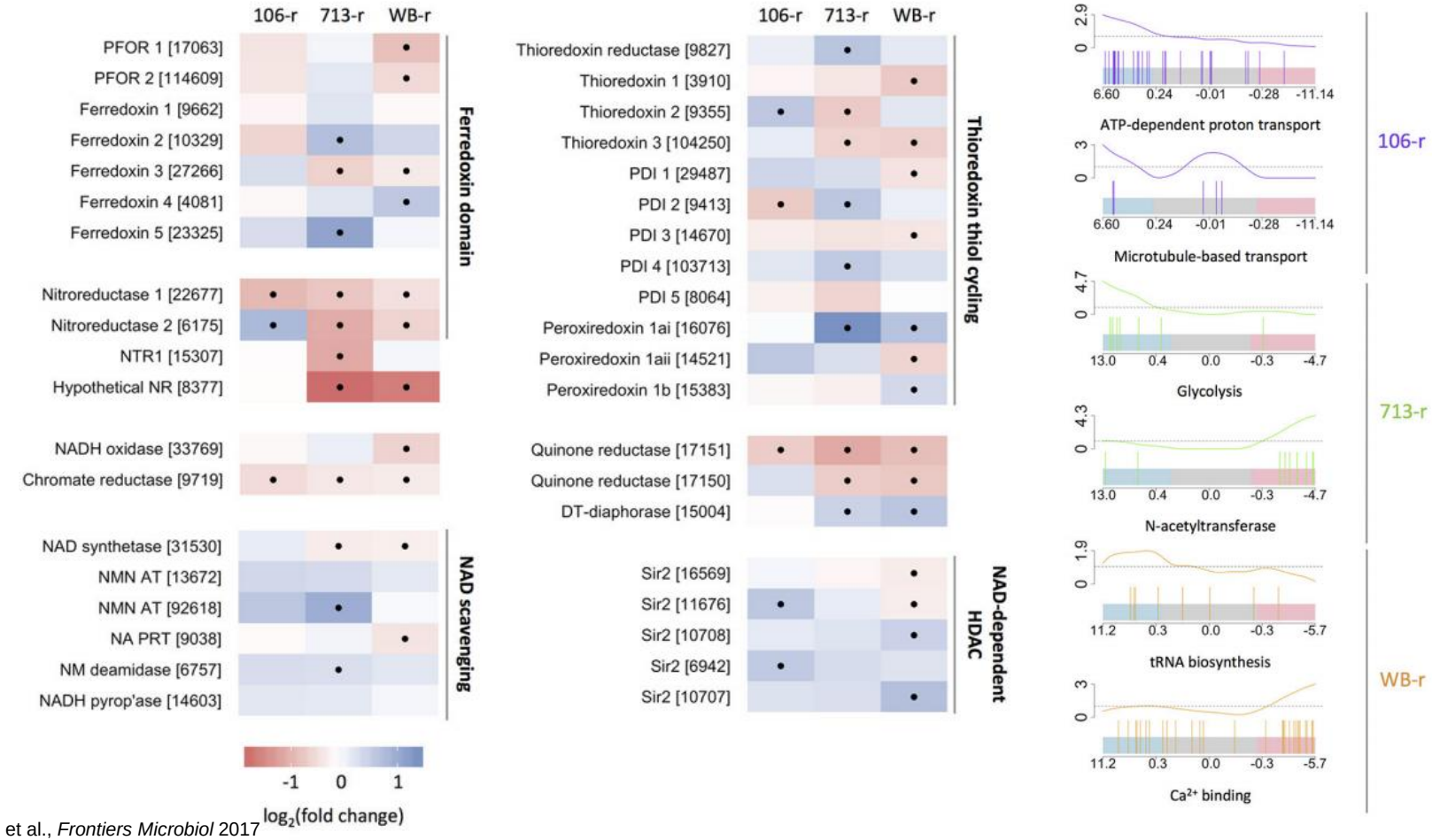
# Resistant *vs* susceptible — common suppressed genes

- Lower carbon flux through PFOR
- Pyruvate accumulation
- Less drug activation




Ansell et al., *Frontiers Microbiol* 2017

# Isotype-specific transcriptional changes




Ansell et al., *Frontiers Microbiol* 2017

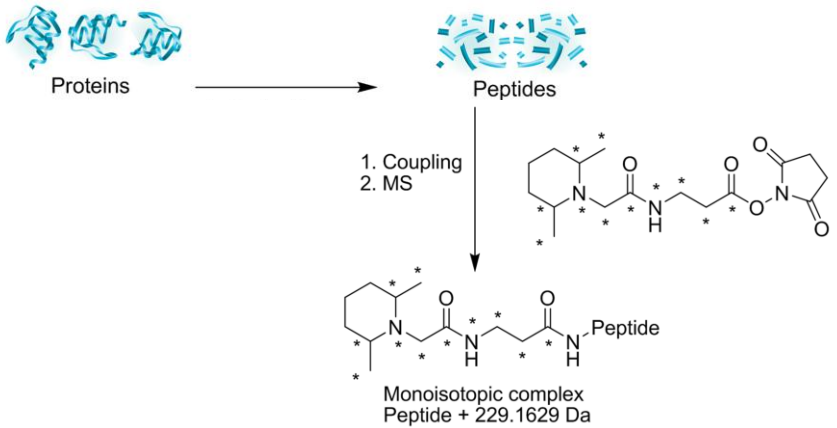
# Isotype-specific mechanisms-specific

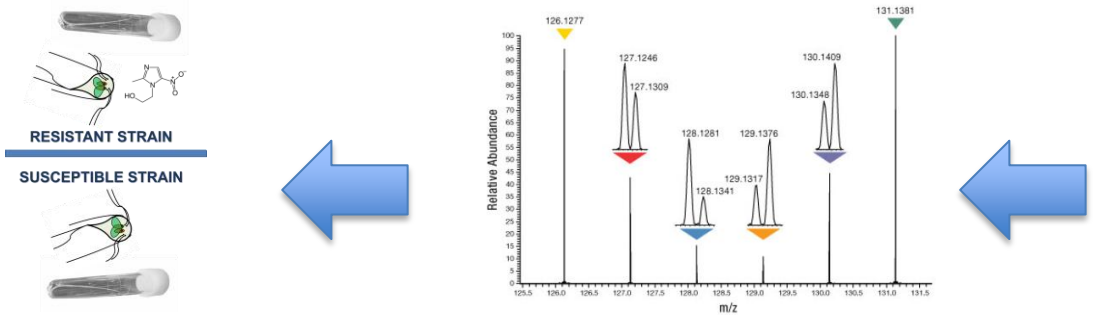
| | Up-regulated (active) | Down-regulated (passive) | |
|---|---|---|---|
| 106-r  | Proton pumps<br>Nitroreductase 2<br>NAD scavenging | | **Drug efflux**<br>**Drug detoxification** |
| 713-r  | Thioredoxin system (thiol cycling)<br>HSPs 90<br>Glycolysis | 2 putative nitroreductases | **Drug activation** ⇄ **Oxidative damage repair**<br>**ATP-driven protein repair (HSPs)** |
| WB-r  | Protein synthesis | Oxygen scavenging<br>PFOR 1, PFOR 2 | **Oxygen accumulation**<br>**Oxidative drug inactivation**<br>**Protein turnover** |

Ansell et al., *Frontiers Microbiol* 2017

# 3-way isogenic isolate (Mtz-R vs Mtz-S) analysis

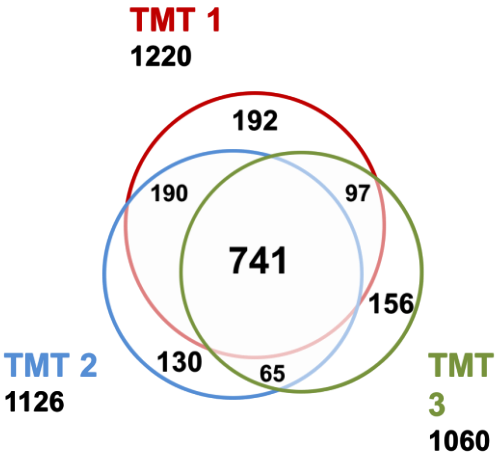
- Isobaric tags
  - TMT Labels
- MS1 Multiplexing, MS2 Quantitation
  - Parallelisation
  - No increased analysis complexity

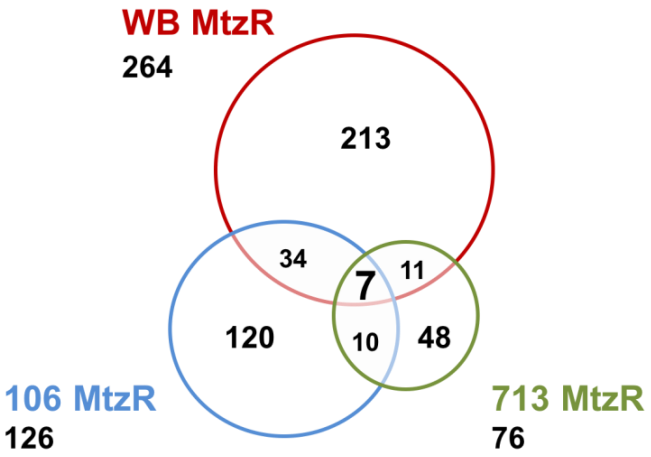


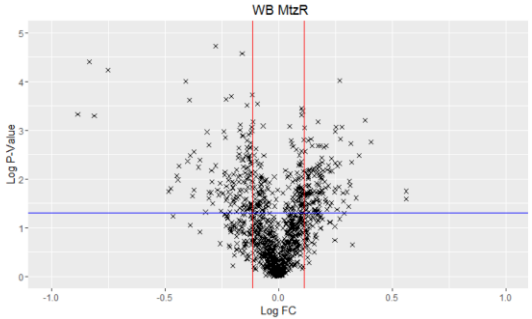

Emery et al., In Review, *Gigascience*

# 3-way isogenic isolate (Mtz-R vs Mtz-S) analysis

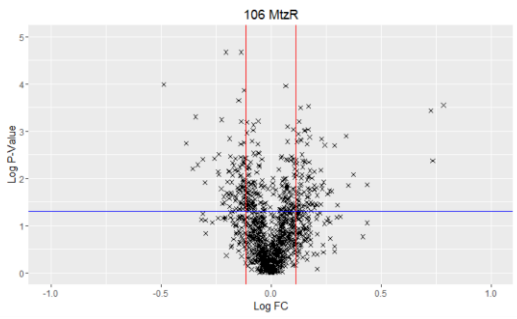


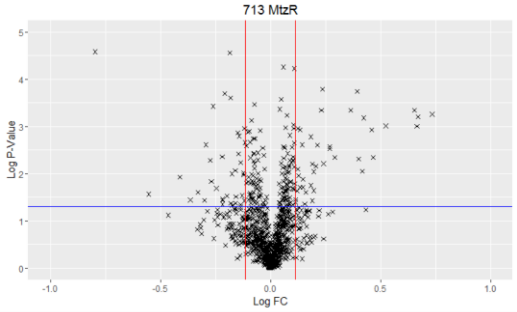

TMT 1 (WB-MtzS vs WB-MtzR)




TMT 2 (106-MtzS vs 106-MtzR)




TMT 3 (713-MtzS vs 713-MtzR)



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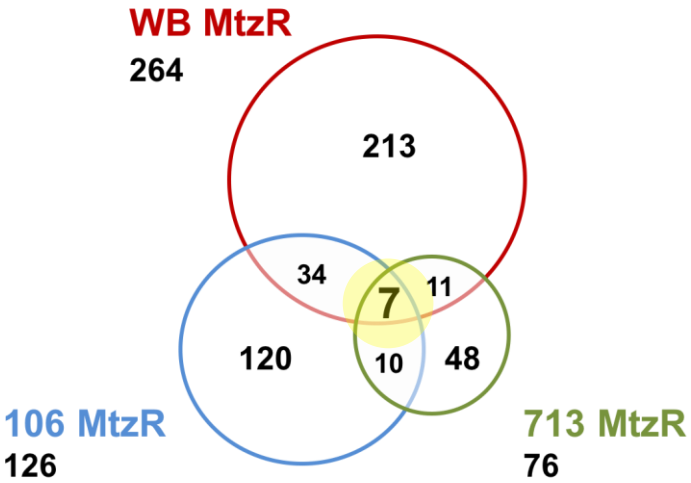
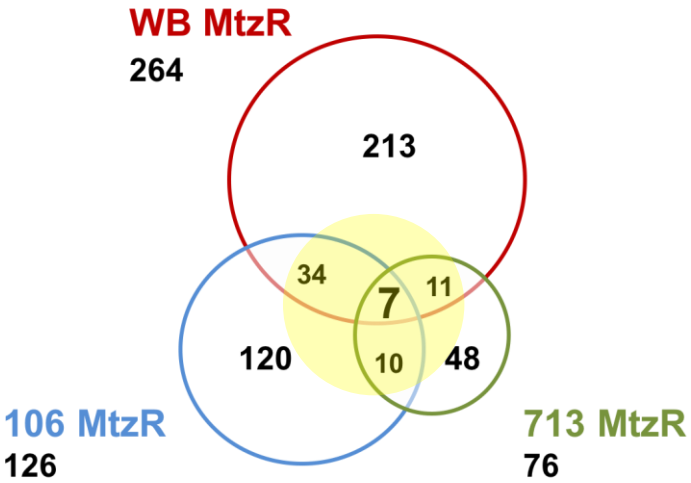
# 3-way isogenic isolate (Mtz-R vs Mtz-S) analysis




- 2 Variant-specific Surface Proteins (VSPs)
- 2 Oxidoreductases
  - Nitroreductase-1 (NR-1)
  - Glutamate Synthase
- 1 EGF transmembrane protein
- 1 membrane-associated endopeptidase

Emery et al., In Review, *Gigascience*

# 3-way isogenic isolate (Mtz-R vs Mtz-S) analysis




- 5 Variant-specific Surface Proteins (VSPs)
- 2 High Cysteine Membrane Proteins (HCMPs)
- 6 Kinases (4 NEK)
- Thioredoxin reductase, Thioredoxin
- 2 membrane-associated endopeptidases

Emery et al., In Review, *Gigascience*

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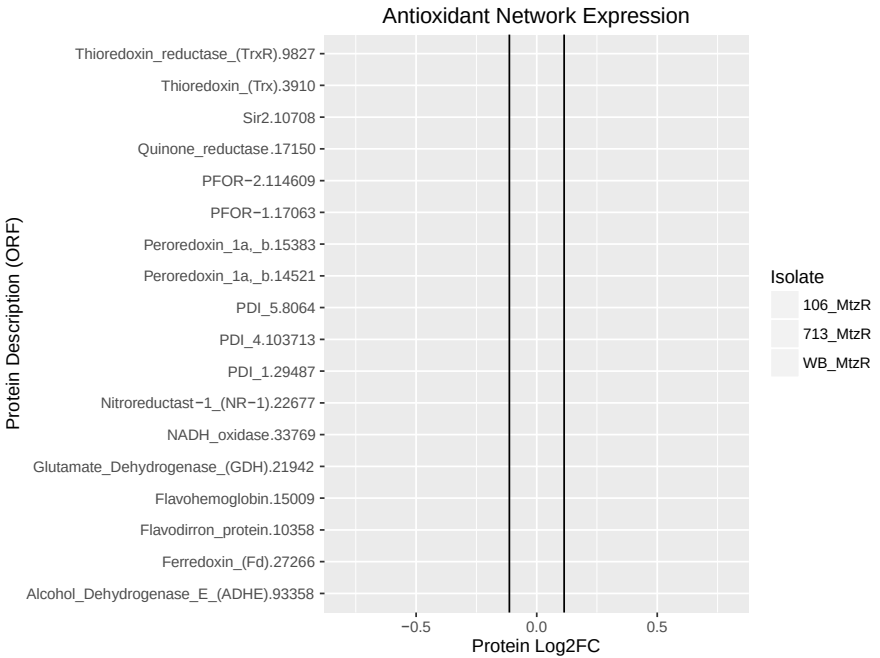
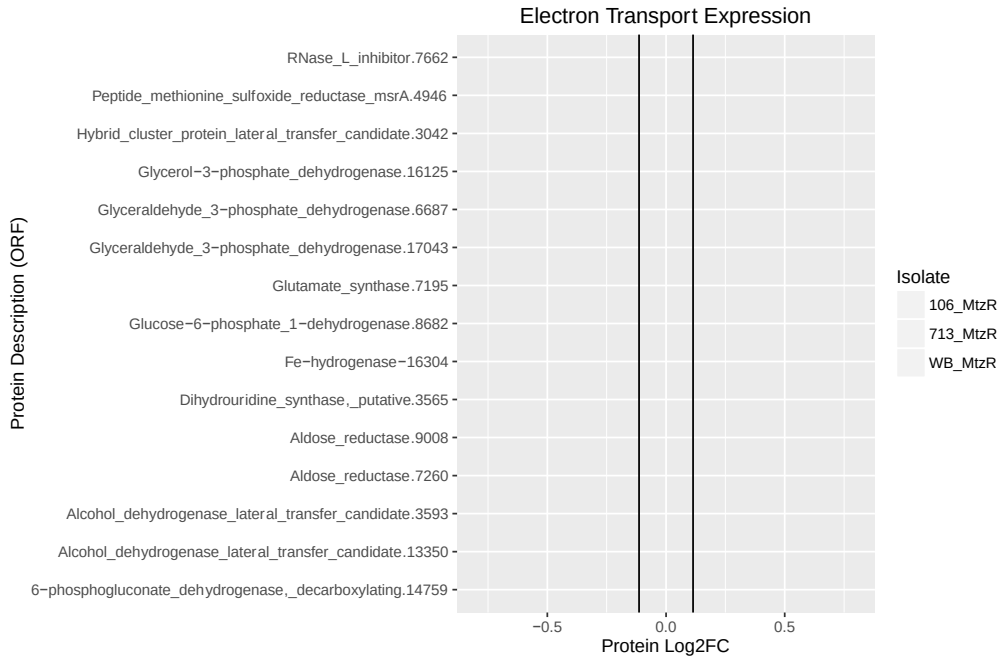
# 3-way isogenic isolate (Mtz-R vs Mtz-S) analysis




Emery et al., In Review, *Gigascience*

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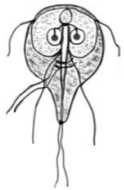
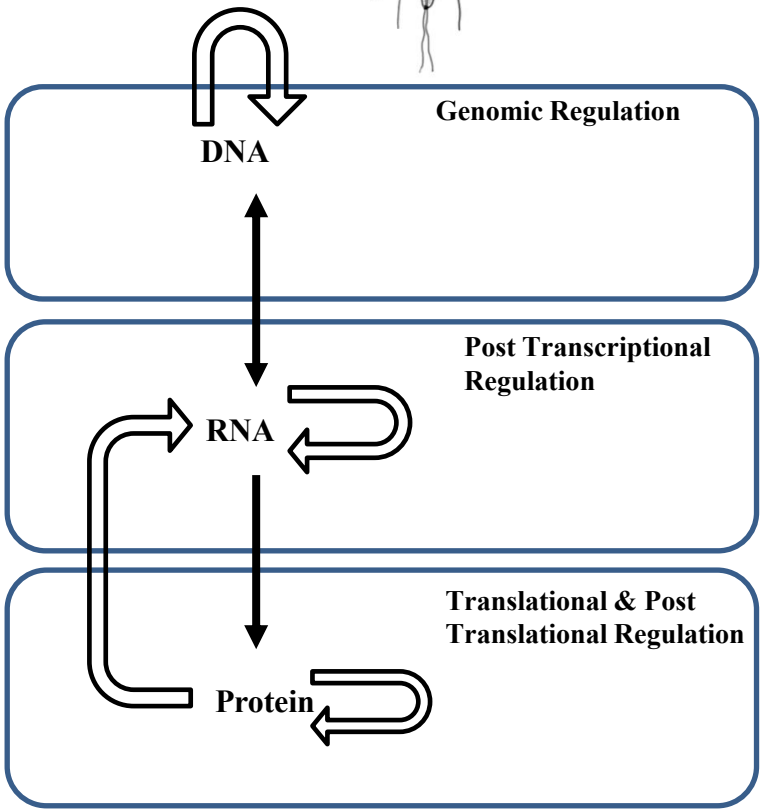
# How are metabolism, stress responses and drug resistance regulated?

# Genetic regulation in *Giardia*






- Minimal Epigentic Components
- Few TFs
- No Regulators Reported
- Leaky Transcription

- RNA Binding Proteins
- RNA Modifications
- RNAi Machinery

- Translational Initiations factors present
- Divergent Translational Initiation

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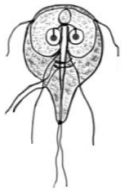
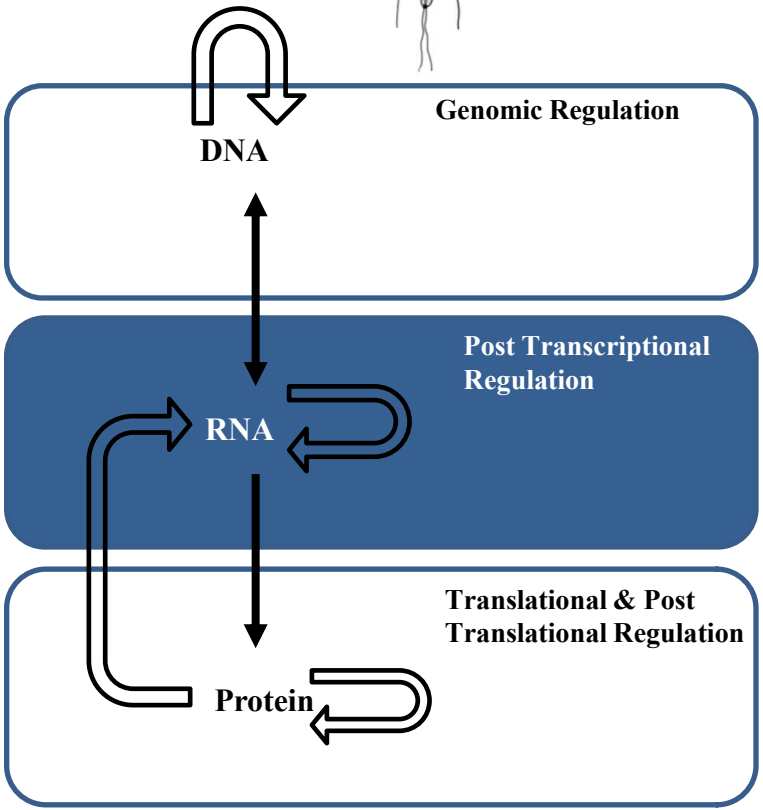
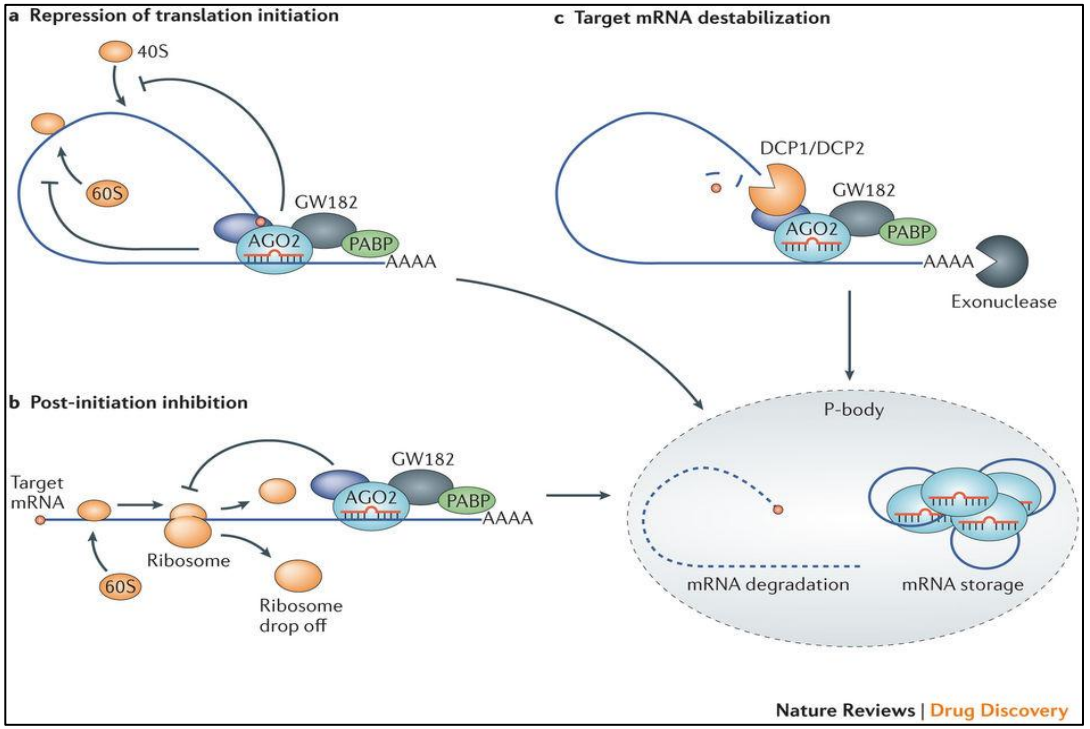
# Post-transcriptional regulation in *Giardia*






Z. Li et al; 2014

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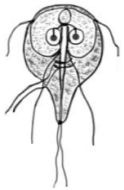
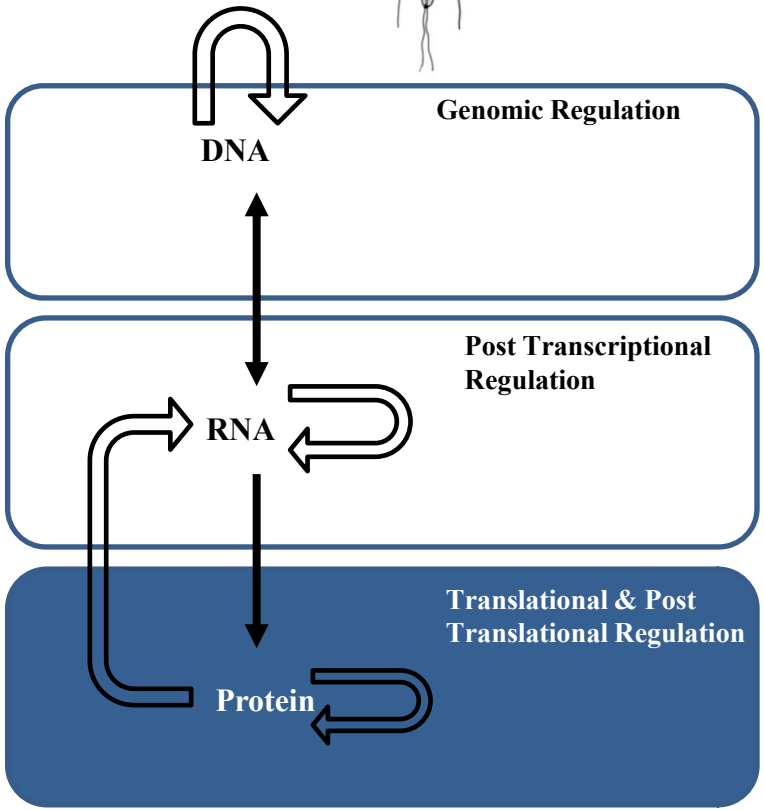
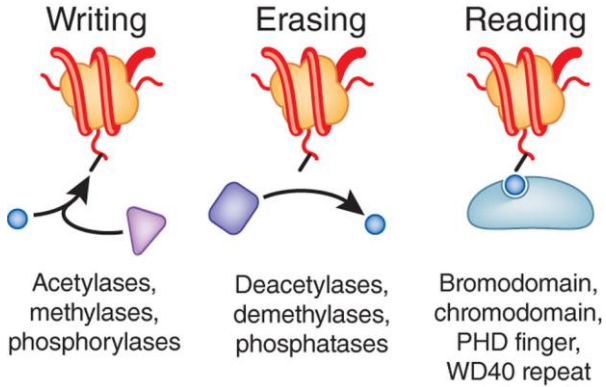
# Post-translational regulation in *Giardia*






- Post-transcriptional regulation
  - Protein post-translational modifications
- MtzR is an unstable/plastic phenotype.
  - Ongoing drug selection
  - Reset during differentiation
- Acetylation in MtzR
  - NAD+-dependent Sirtuins
  - *Muller et al, 2008*
  - *Ansell et al, 2015*

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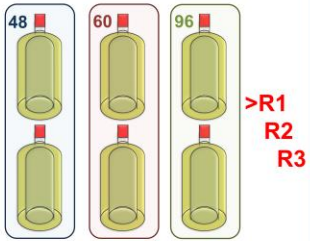
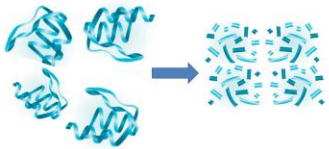
# KAc/KMe experimental workflow

## 1. Sample Preparation

**Giardia Culture (WB-1B)**
Axenic culture, TYI-S33, Microaerophilic



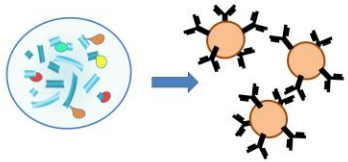

**Cell harvest and lysis**
8M Urea, Probe Sonication



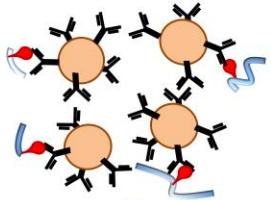
**Protein extraction & digestion**
Lys-C Pre-digestion, Trypsin digestion,
C18 SepPak Cleanup

## 2. IAP Enrichment

**Tryptic Peptides**



**Anti-acetyllysine Multi-MAb**
**Anti-methylysine Multi-MAb**
**(PTMScan, CST)**

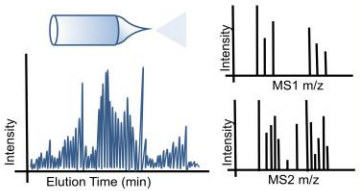


**Enriched KAc/KMe* peptides**
SDB-RPS SPE

***Secondary KMe digestion***
Trypsin; SDB-RPS SPE

## 3. LC-MS/MS Analysis

**OrbiTrap Fusion Lumos**
HCD, 21min gradient, Tech Rep Injections

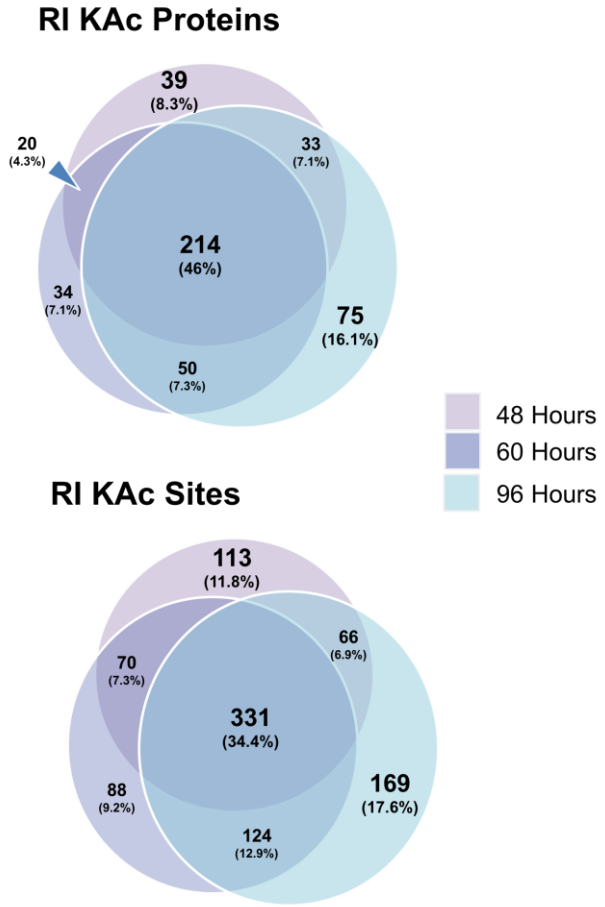



**Computational Analysis**
Database searching

**Bioinformatics**

# KAc results summary

- 896 KAc proteins/2382 NR sites (n=2)
- 12.1% non-deprecated genes



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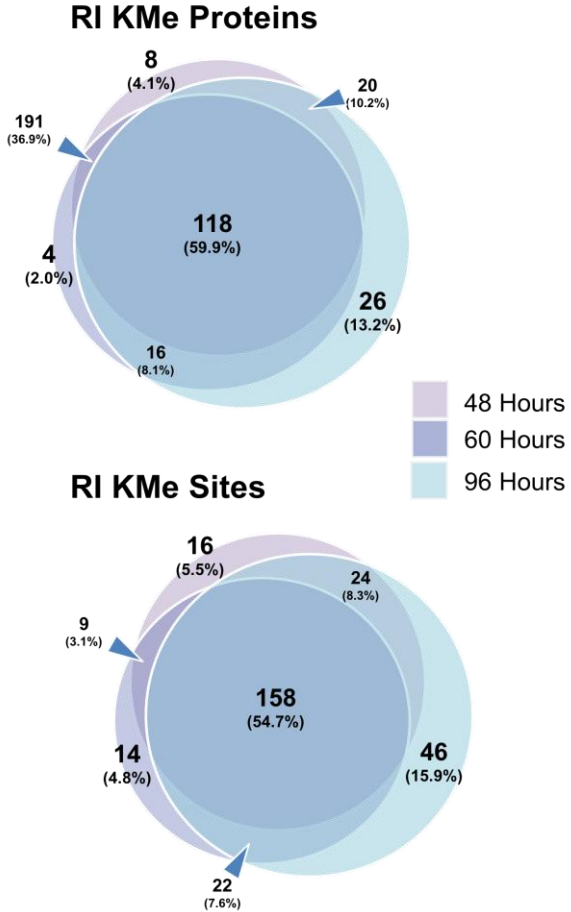
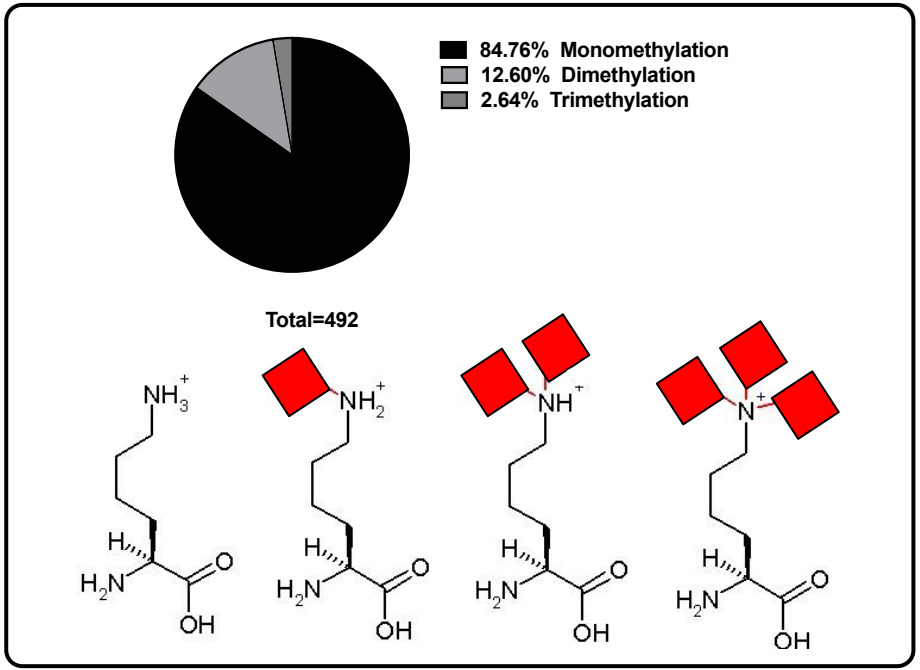
# KMe results summary




- 304 KMe proteins/492 NR sites (n=2)
- Majority mono-methylated



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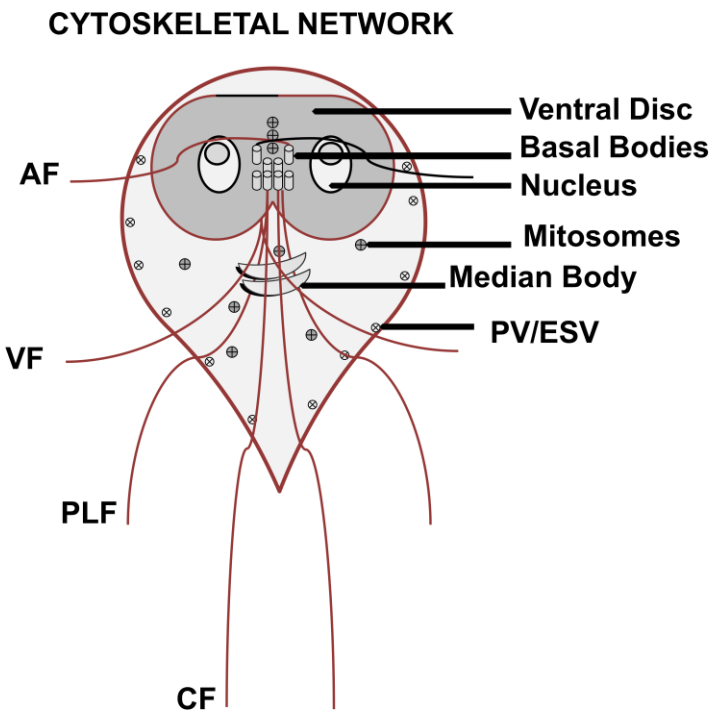
# KAc/KMe Subcellular Distribution




- Ac — Giardins
  - 15 alpha-giardins
  - Beta-, delta-, gamma-giardin
- Ac Me — Ankyrin-repeat/coiled-coil
  - NEK kinases
  - Protein 21.1
- Ac Me — Kinesins
- Ac Me — Dyneins
- Ac Me — Intraflagella Transport (IFT) Proteins
- Ac Me — Axoneme-associated proteins

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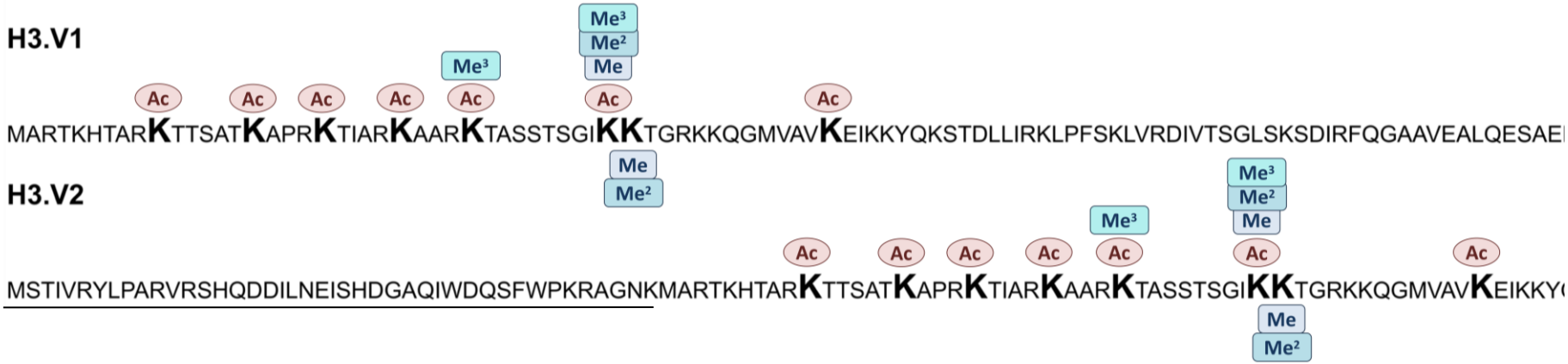
# Histone modification sites



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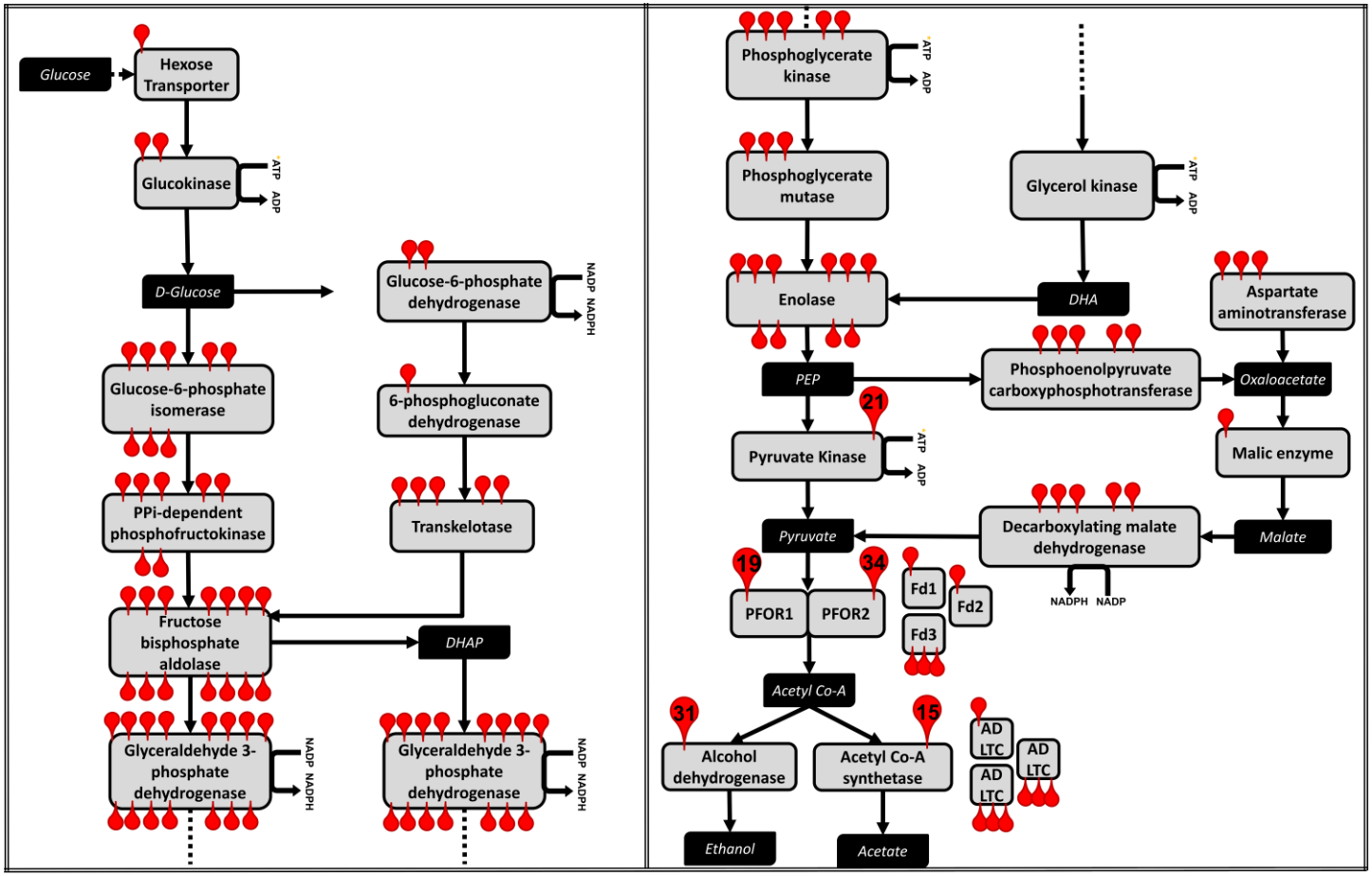
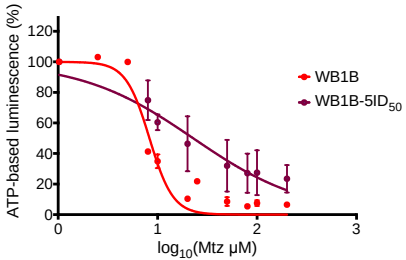
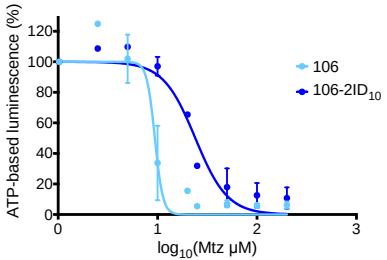
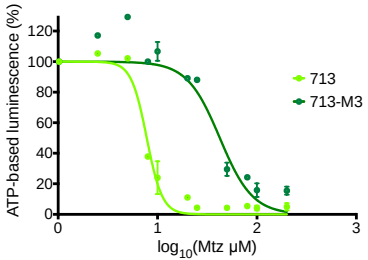
# KAc in Energy Metabolism

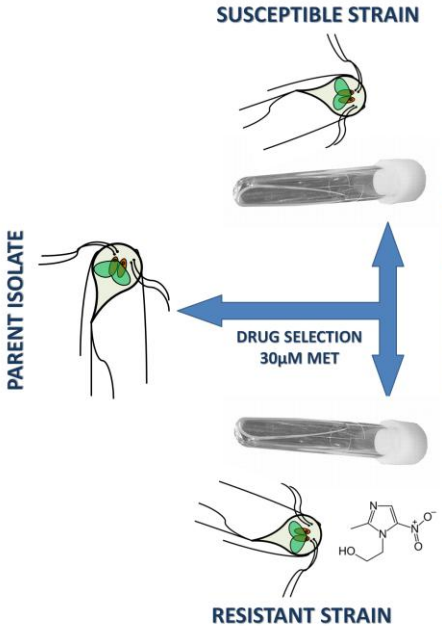


# 3-way isogenic isolate (Mtz-R vs Mtz-S) analysis



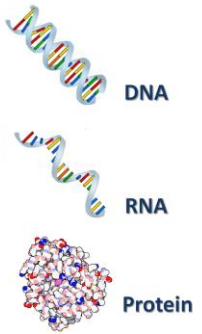




TRIPURE (ROCHE) WHOLE TROPHOZOITE EXTRACTION

IC50 DETERMINATION-- CELLTITRE-GLO ATP-BASED LUMINESCENCE ASSAY

| Isolate | Sus IC50 | Res IC50 | Resistance Factor (RF) |
|---|---|---|---|
| WB | 8.28 µM | 22.79 µM | 2.8 |
| 106 | 9.39 µM | 23.99 µM | 2.6 |
| 713 | 7.79 µM | 42.33 µM | 5.4 |




TRIPURE (ROCHE) WHOLE TROPHOZOITE EXTRACTION

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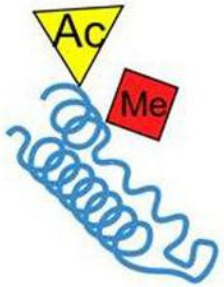
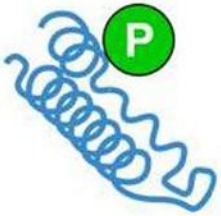
# 3-way isogenic isolate (Mtz-R vs Mtz-S) analysis

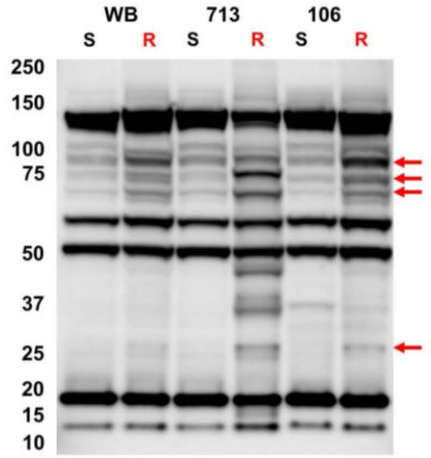



**Lysine Acetylation**
**Lysine Methylation**

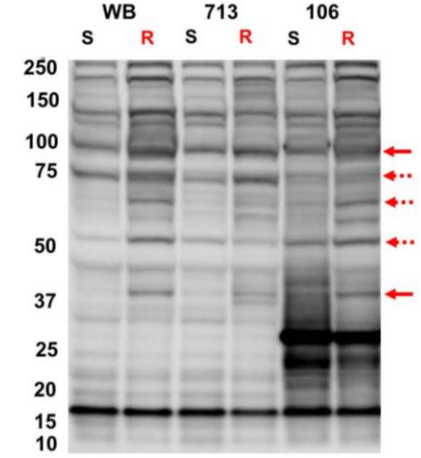



**Serine/Threonine & Tyrosine Phosphorylation**

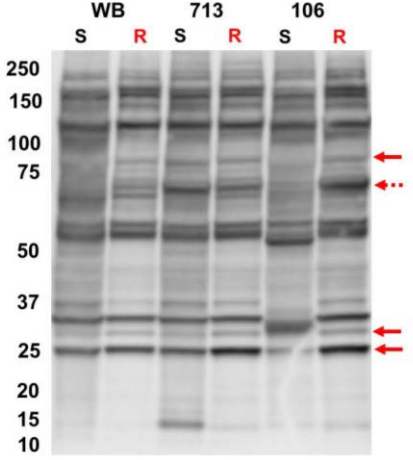
**KAc**



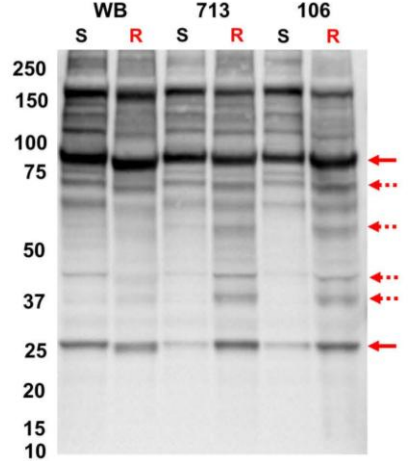

**K-MMe**




**pY**




**14-3-3**



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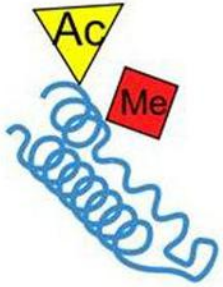
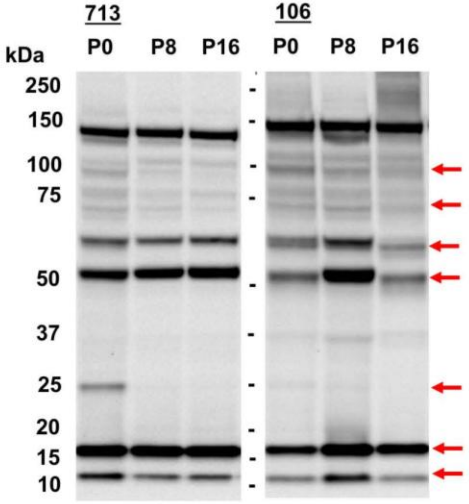
# 3-way isogenic isolate (Mtz-R vs Mtz-S) analysis



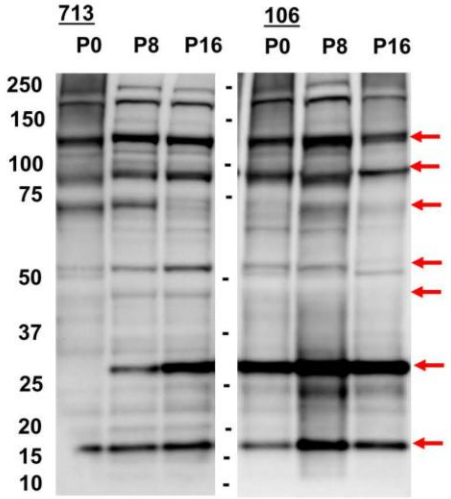

**Lysine Acetylation**
**Lysine Methylation**


**Serine/Threonine & Tyrosine Phosphorylation**

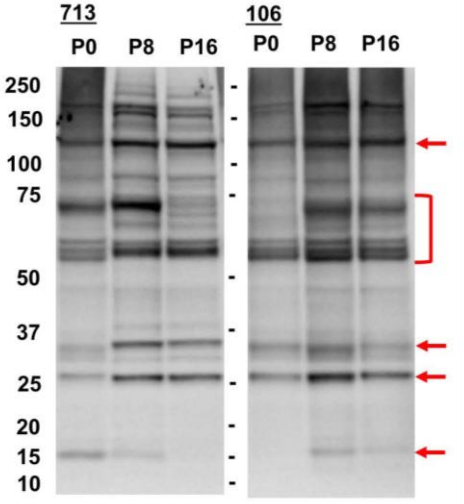
**i) KAc**




**ii) K-MMe**




**iii) pY**



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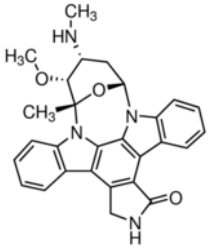
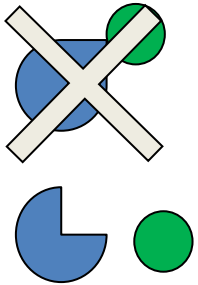
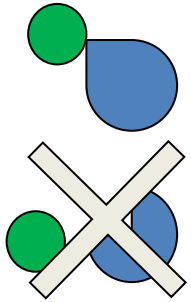
# 3-way isogenic isolate (Mtz-R vs Mtz-S) analysis

## Staurosporine

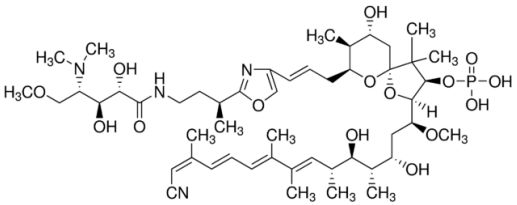




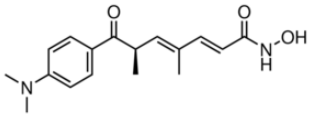
## Serine/Threonine & Tyrosine Phosphorylation

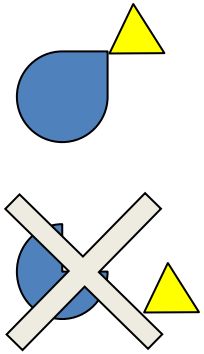



## Calyculin A

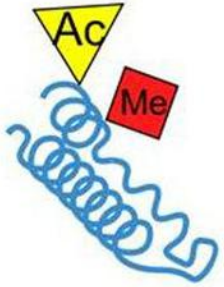


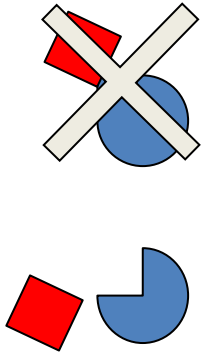
## Trichostatin A (TSA)



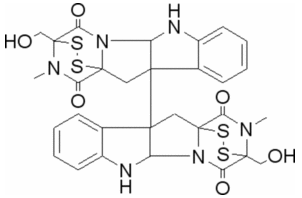


## Lysine Acetylation Lysine Methylation



## Chaetocin



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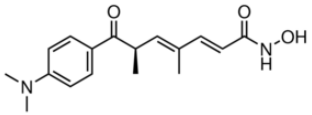
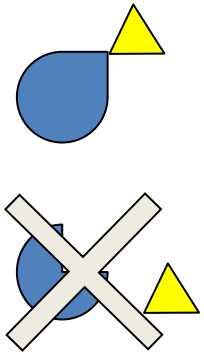
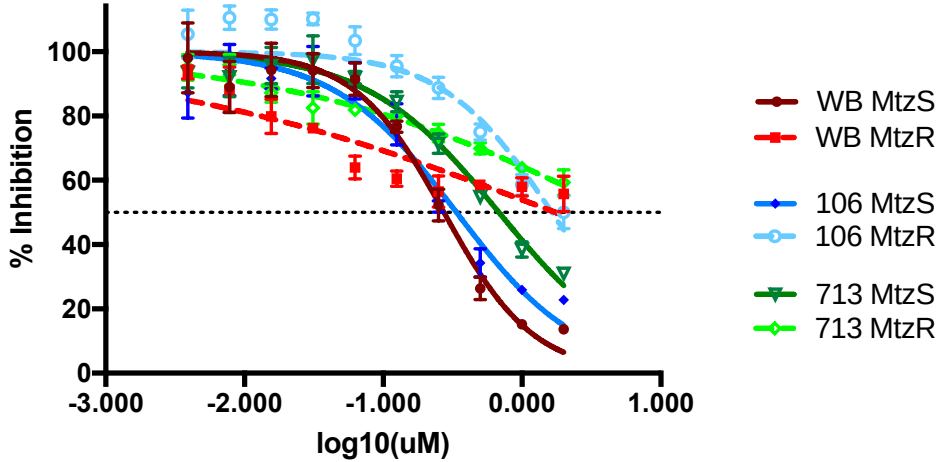
# 3-way isogenic isolate (Mtz-R vs Mtz-S) analysis

**Trichostatin A (TSA)**







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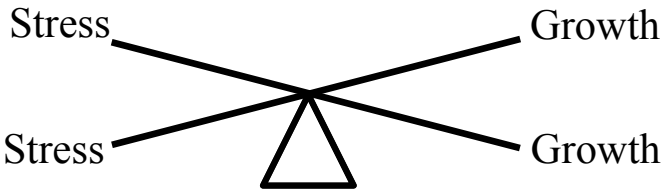
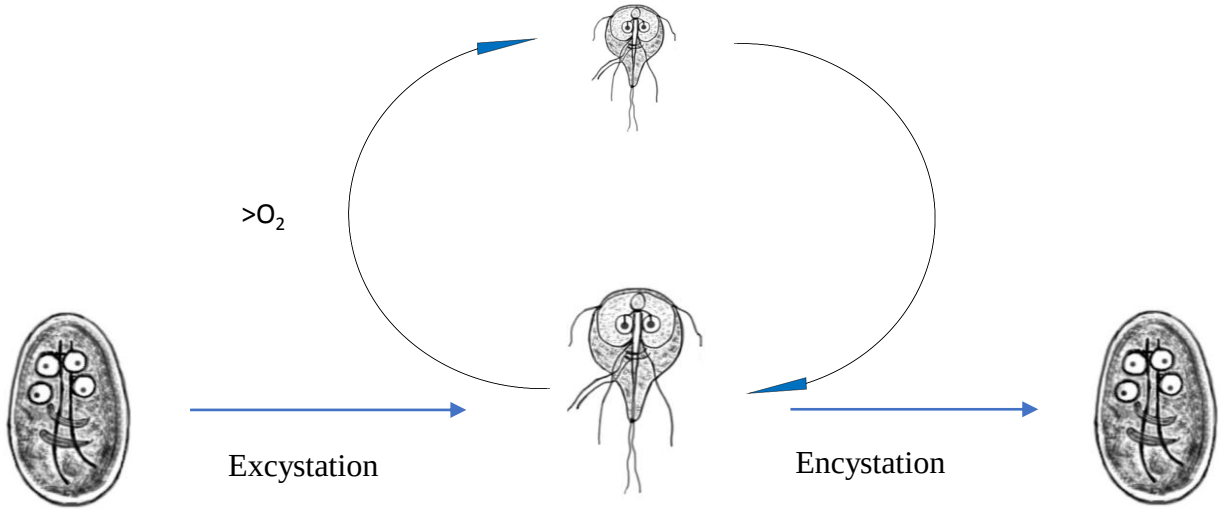
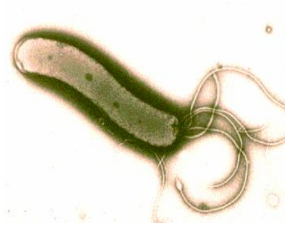
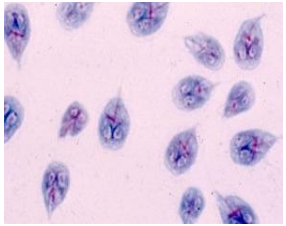
# Is managing stress key to *Giardia* growth and Mtz-resistance?

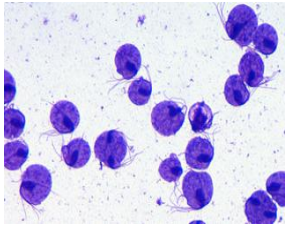


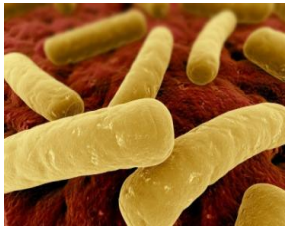



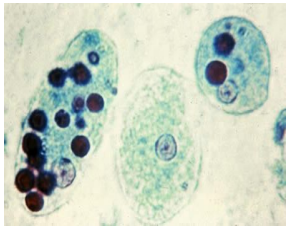


*Helicobacter pylori*


*Giardia duodenalis*


*Bacteriodes fragilis*


*Trichomonas vaginalis*


*Clostridium sp.*


*Entamoeba histolytica*

# Next steps

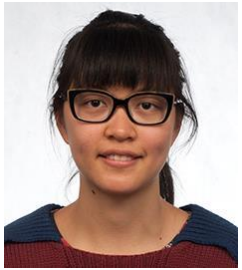
- Transcription vs translation – regulatory mechanisms – Balu Balan
- Role of PTMs in metabolism, Mtz resistance and stress response – Sam Emery
- Are there core/universal responses to multi-drugs or multi-stresses?
- Are stress responses adaptively encoded in the cyst?
- Can these be targeted to limit resistance?


Brendan


Louise


Nijoy


Sa


Jo


Katharina


Balu

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  - Daniel Vuong
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- Uppsala University
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  - Showgy Ma'ayeh
  - Elin Elinarsson
- YourGene Biosciences
  - Bill Chang

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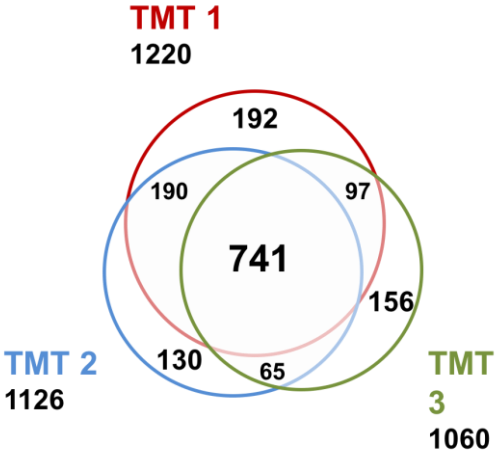
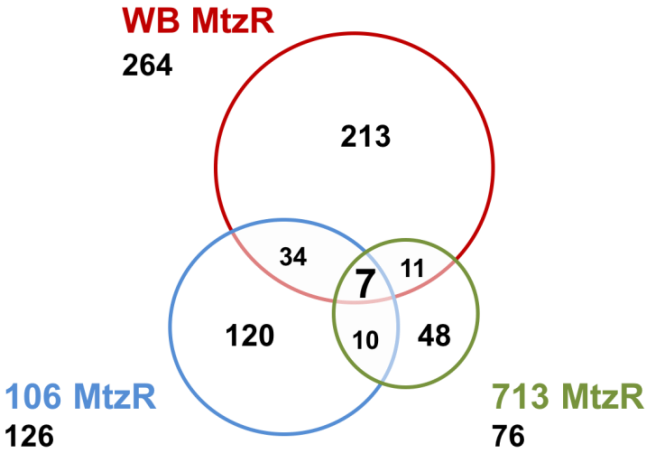
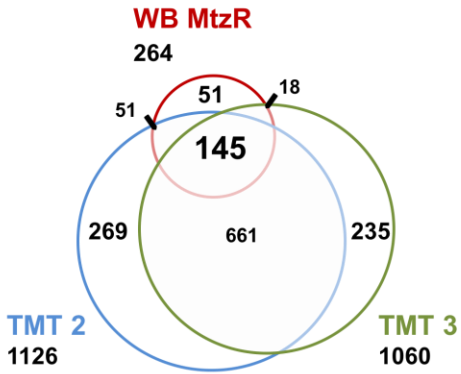
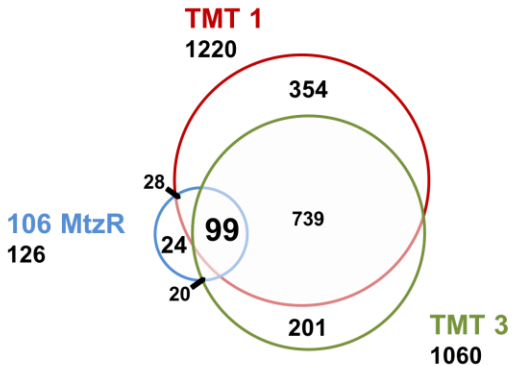
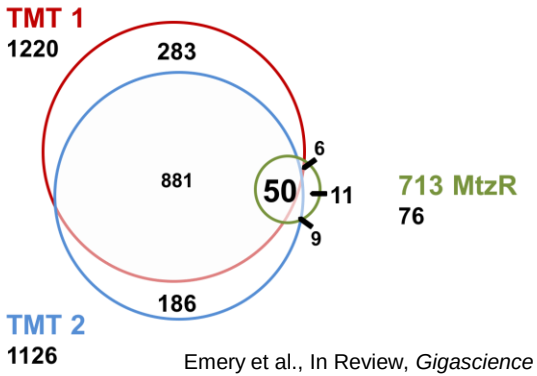
# 3-way isogenic isolate (Mtz-R vs Mtz-S) analysis




Emery et al., In Review, *Gigascience*

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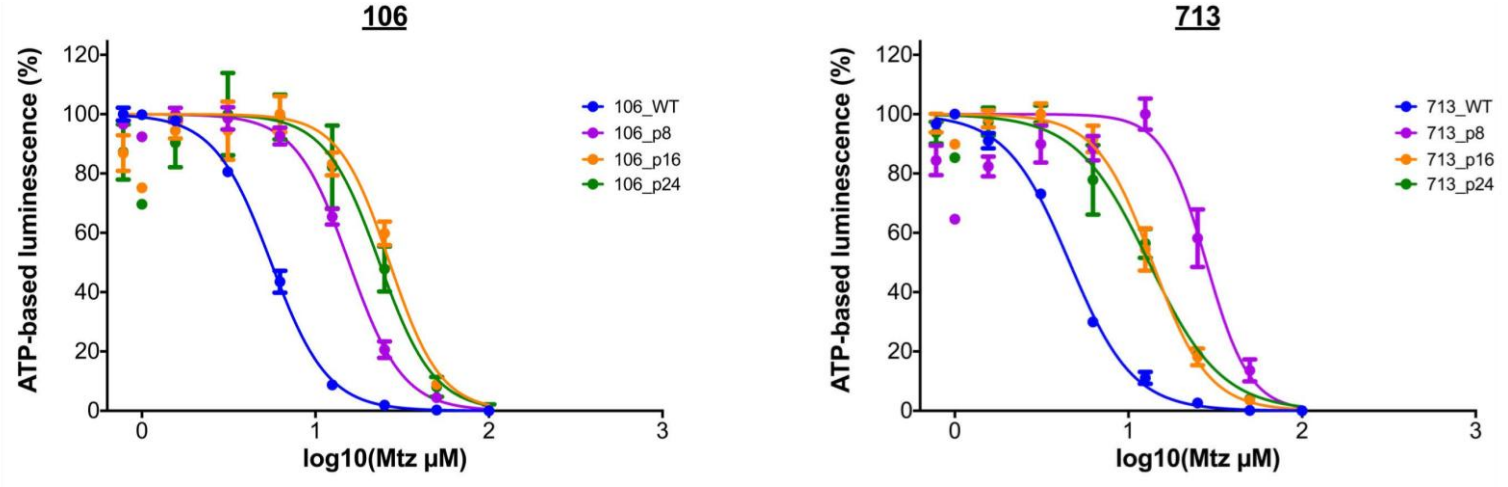


| | WT | P8 | | P16 | | P24 | |
|---|---|---|---|---|---|---|---|
| | Mtz $IC_{50}$ (µM) | Mtz $IC_{50}$ (µM) | RF | Mtz $IC_{50}$ (µM) | RF | Mtz $IC_{50}$ (µM) | RF |
| **106** | 5.5 | 15.6 | 2.8 | 26.7 | 4.8 | 23.4 | 4.2 |
| **713** | 4.6 | 28.1 | 6.1 | 13.7 | 3.0 | 13.1 | 2.9 |