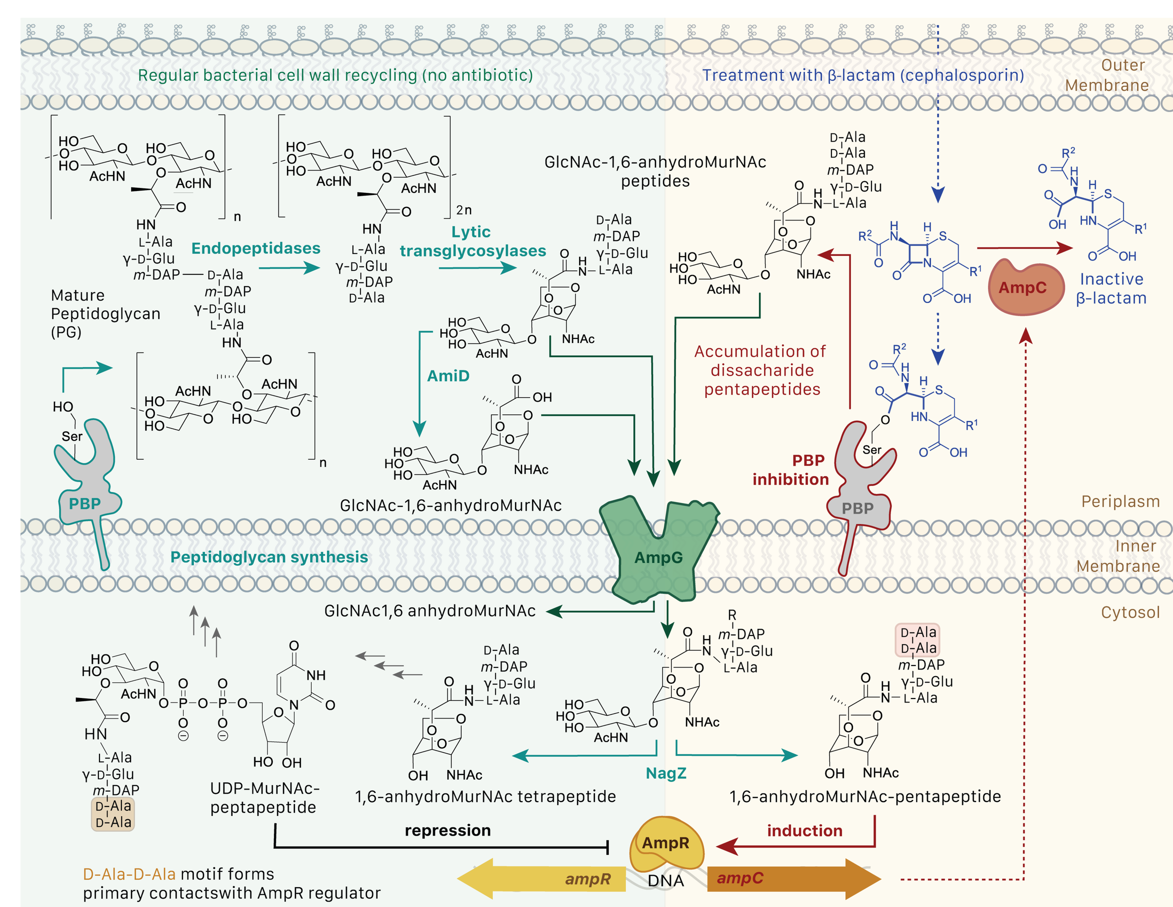
Cryo-EM characterization of the anydromuropeptide permease AmpG central to bacterial fitness and β-lactam antibiotic resistance

Sverak HE[1,2], Yaeger LN[3], Worrall LJ[1,2,4], Vacariu CM[5], Glenwright AJ[1,2], Vuckovic M[1,2], Al Azawi ZD[3], Lamers RP[3], Marko VA[3], Skorupski C[3], Soni AS[5], Tanner ME[5], Burrows LL[3], Strynadka NCJ[1,2,4,*]

[1]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada
[2]Centre for Blood Research, University of British Columbia, Vancouver, BC, Canada
[3]Department of Biochemistry and Biomedical Sciences and the Michael G. DeGroote Institute of Infectious Disease Research, McMaster University, Hamilton, Ontario, Canada
[4]High Resolution Macromolecular Cryo-Electron Microscopy (HRMEM) Facility, University of British Columbia, Vancouver, BC, Canada
[5]Department of Chemistry, University of British Columbia, Vancouver, BC, Canada

Introduction

Antimicrobial resistance is an escalating global health crisis. Understanding current resistance mechanisms enables the creation of cocktail therapeutics, prolonging effectiveness of existing antibiotics. One mechanism of concern is AmpC, an enzyme that inactivates nearly all β-lactam antibiotics[1]. AmpC production is the primary driver of β-lactam resistance in *P. aeruginosa*[2] and causes broad spectrum β-lactam resistance in *K. pneumoniae* and *Enterobacter*[3,4].



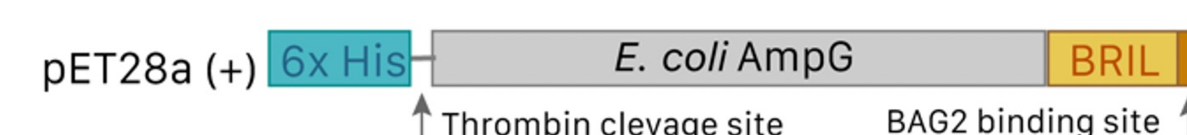
A complex signaling system of cell wall fragments controls AmpC expression. Mature peptidoglycan (PG) is typically processed into GlcNAc-1,6-anhydroMurNAc peptides and recycled across the inner membrane, enhancing bacterial fitness. β-lactam treatment disrupts this, causing accumulation of PG peptide fragments, which induce *ampC* expression. PG fragment transport is facilitated by the essential major facilitator superfamily (MFS) importer **AmpG**. Δ*ampG* mutants regain β-lactam sensitivity and have inhibited biofilm formation.[5]

Objective

Characterization of AmpG atomic structure, substrate binding, and mechanism of action to understand this process in bacteria and its potential druggability to combat AmpG-mediated β-lactam resistance in serious pathogens.

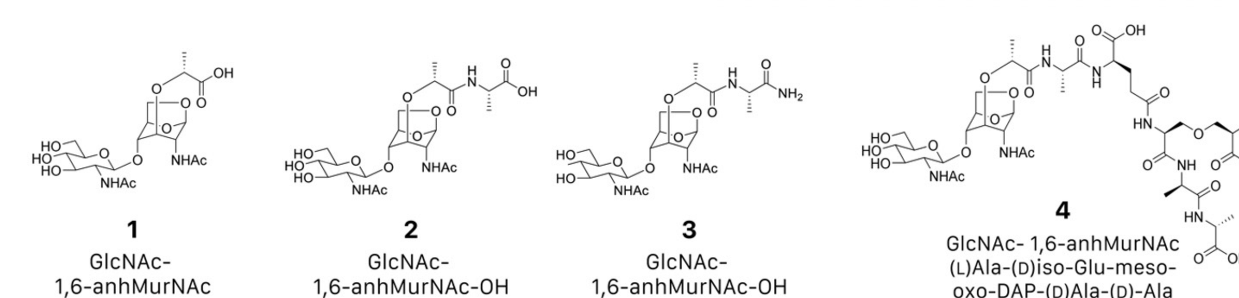
Methods

Wild-Type (WT) *E. coli* AmpG was expressed and screened for optimized purification conditions. Structural determination of this small ~53 kDa membrane protein was assisted with the addition of apocytochrome b562 (BRIL), which can be used as an epitope for the synthetic antibody BAG2[6].



Substrate analog chemical synthesis

Performed by the Tanner lab in the Department of Chemistry at the University of British Columbia

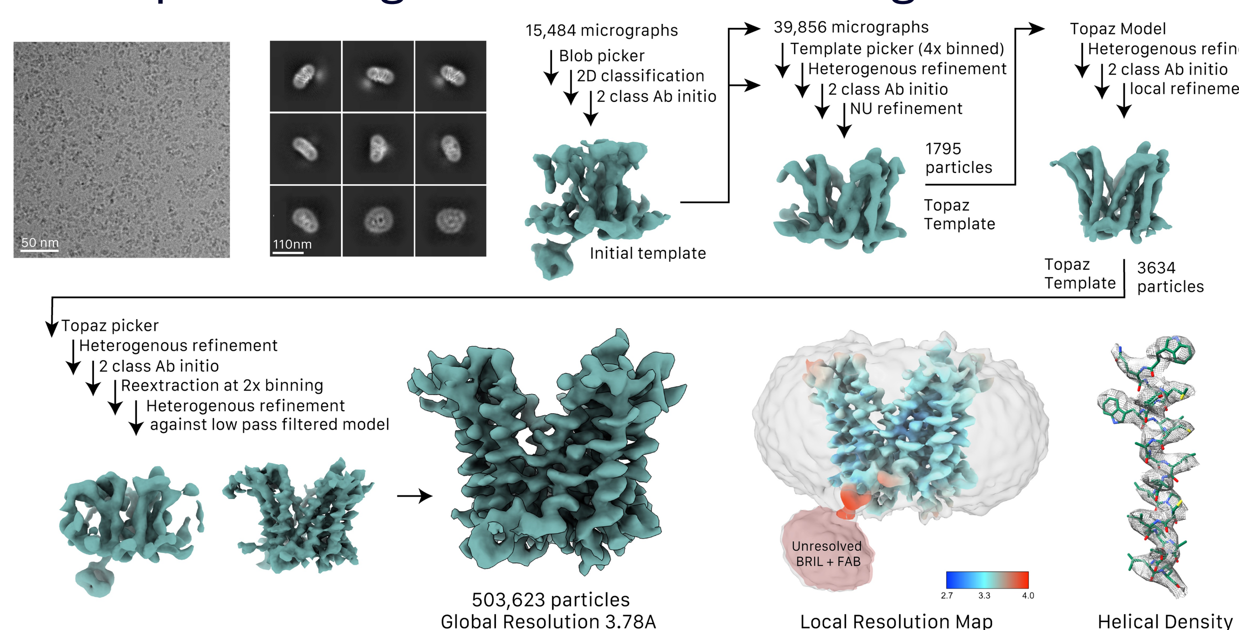


Cellular Cytotoxicity and mutagenesis

Cellular minimum inhibitory concentration assays (MIC) in a *P. aeruginosa* expression system were performed by our collaborators in the Burrows lab at the University of McMaster. A Δ*ampG* PAO1 strain was used to test *E. coli and P. aeruginosa* AmpG WT and mutant activity for cefoxitin MIC. Reverse genetic screens were performed with mutants to find rescue mutations of interest.

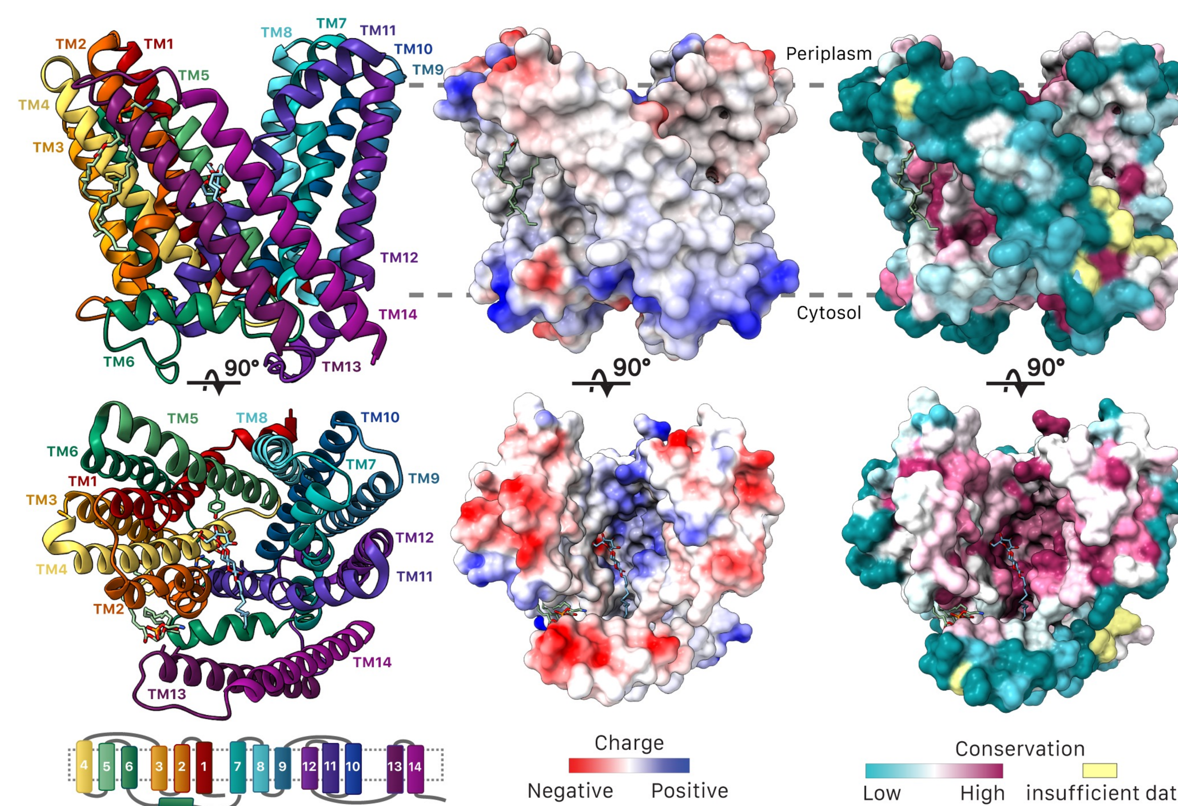
Binding Assays

Isothermal calorimetry (ITC) and microscale thermophoresis (MST) was used for characterization of AmpG binding with substrate analogs.

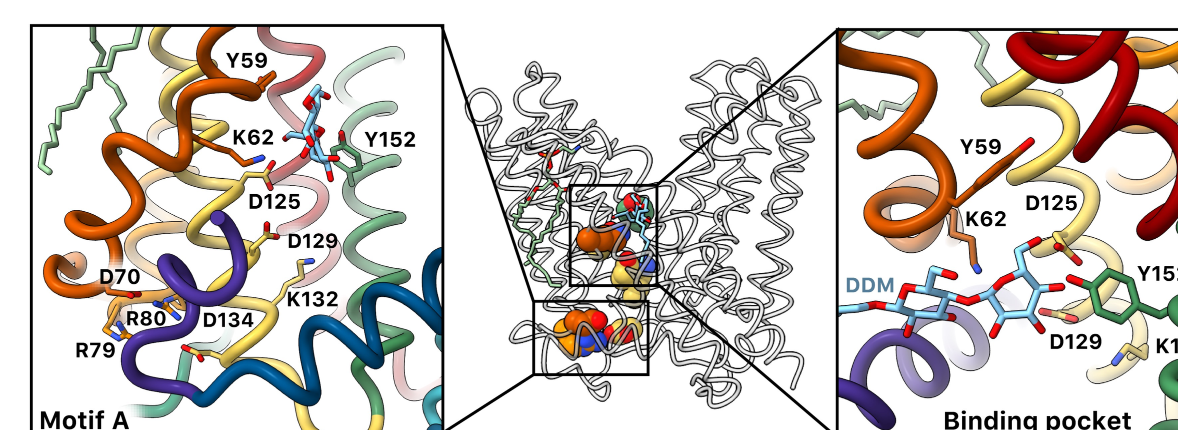


Results

Cryo-EM structure of WT *E. coli* AmpG

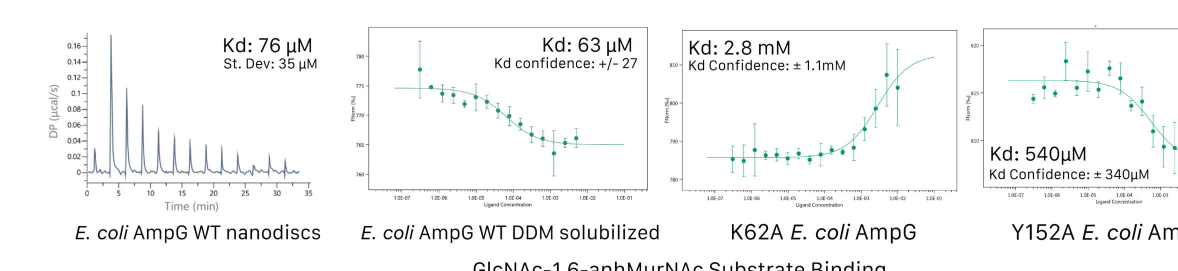


AmpG is stabilized in an outward-open conformation composed of 14 transmembrane helices. A well-known MFS motif (motif A) stabilizes the open confirmation, acting as a molecular latch, with Asp70 as the central residue.



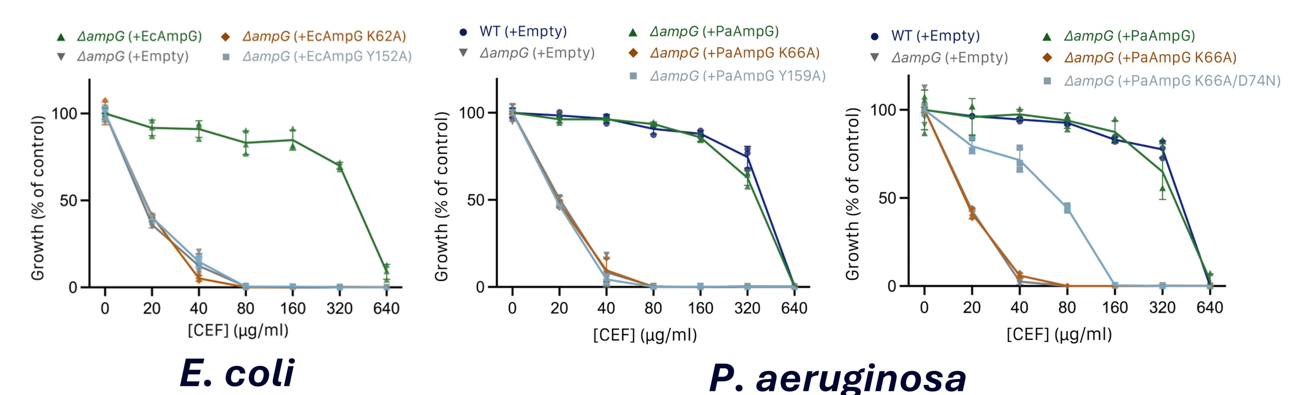
AmpG displays a large binding cavity, with density corresponding to a DDM bound. We propose the sugar moieties of the DDM mimic the binding of the substrate GlcNAc-1,6-anhydroMurNAc, interacting with highly conserved residues Lys62, Asp125, Asp129, and Tyr152. K62 and Y152 were chosen for mutational analysis

In vitro analysis of AmpG substate binding



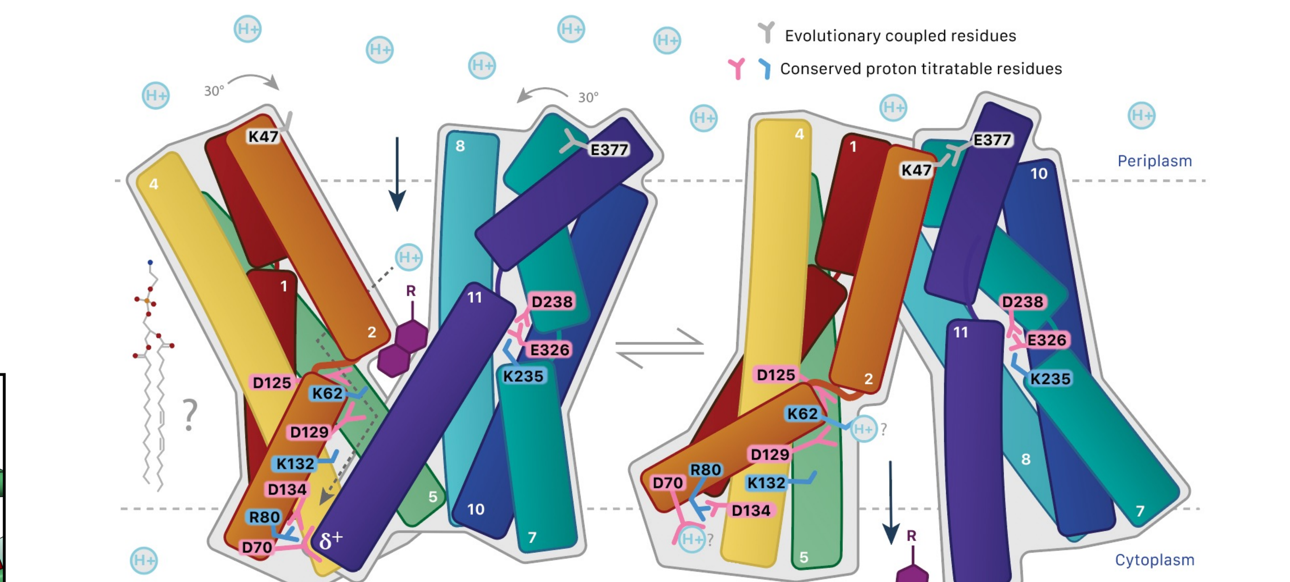
ITC and MST of AmpG demonstrated similar binding values of GlcNAc-1,6-anhydroMurNAc with literature[8]. Alanine mutants of K62 and Y152 both substantially lowered binding affinity.

In vivo analysis of AmpG in a *P. aeruginosa* system



Effect of cefoxitin on the growth of a *P. aeruginosa* Δ*ampG* strain. Expression of *E. coli* WT AmpG restored growth, while mutants K62A and Y152A did not. In *P. aeruginosa* homologs (K66A, Y159A), the same effect was shown. In K66A, a D74N (E. coli D70 analog) mutant was identified that rescued growth.

Proposed mechanism of action of AmpG



Conclusions

- **Cryo-EM Characterization**: Structure of *E. coli* AmpG with a defined substrate binding cavity.
- **Binding Determinants:** Disaccharide moiety seems to be the primary factor in substrate binding, supported by transport assays[8].
- **Impact of AmpG Deletion:** Deletion from PAO1 strains resulted in a 30-fold decrease in MIC.
- **Mutation Effects:** Mutations in binding pocket residues Lys62 and Tyr152 abolished activity, indicating the potential for an inhibitor
- **Therapeutic Strategy:** Targeting the AmpG symporter is promising for developing synergistic antimicrobials to enhance efficacy.

Acknowledgments

We thank Professor Anthony Kossiakoff from the University of Chicago for the generous gift of BRIL and BAG2 constructs. We acknowledge infrastructure support from the Canadian Foundation of Innovation and BC Knowledge Development Fund, and the High-Resolution Macromolecular EM facility at UBC for assistance with grid screening and data collection.

THE UNIVERSITY OF BRITISH COLUMBIA
Department of Biochemistry & Molecular Biology
Faculty of Medicine

References

1. Tamma, P. D., Doi, Y., Bonomo, R. A., Johnson, J. K. & Simner, P. J. A Primer on AmpC β-Lactamases: Necessary Knowledge for an Increasingly Multidrug-resistant World. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* **69**, 1446–1455 (2019).
2. Torrens, G. *et al.* Regulation of AmpC-Driven β-Lactam Resistance in Pseudomonas aeruginosa: Different Pathways, Different Signaling. *mSystems* **4**, e00524-19 (2019).
3. D'Souza, R. *et al.* Role of AmpG in the resistance to β-lactam agents, including cephalosporins and carbapenems: candidate for a novel antimicrobial target. *Ann. Clin. Microbiol. Antimicrob.* **20**, 45 (2021).
4. Barceló, I. M. *et al.* Filling knowledge gaps related to AmpC-dependent β-lactam resistance in Enterobacter cloacae. *Sci. Rep.* **14**, 189 (2024).
5. Zamorano, L. et al. AmpG inactivation restores susceptibility of pan-beta-lactam-resistant Pseudomonas aeruginosa clinical strains. Antimicrob. Agents Chemother. 55, 1990–1996 (2011).
6. Mukherjee, S. et al. Synthetic antibodies against BRIL as universal fiducial marks for single−particle cryoEM structure determination of membrane proteins. Nat. Commun. 11, 1598 (2020).
7. Punjani, A., Rubinstein, J. L., Fleet, D. J. & Brubaker, M. A. cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. Nat. Methods 14, 290–296 (2017).
8. Perley-Robertson, G. E. et al. A Fluorescent Transport Assay Enables Studying AmpG Permeases Involved in Peptidoglycan Recycling and Antibiotic Resistance. ACS Chem. Biol. 11, 2626–2635 (2016).

Funding

INNOVATION Canada Foundation for Innovation / Fondation canadienne pour l'innovation
CBR
NSERC CRSNG
hhmi Howard Hughes Medical Institute
CIHR IRSC