## **Supplementary Information and Materials and methods**

# Evidence for [2Fe-2S]<sup>2+</sup> and linear [3Fe-4S]<sup>1+</sup> clusters in a unique family of glycine/cysteine-rich Fe-S proteins from *Megavirinae* giant viruses

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# **Supplementary Information**



**Fig S1: (A)** Bacterial pellets (*E. coli* Rosetta strain) overproducing mimivirus tagged (His<sub>6</sub>-SUMO) GciS (left) and His<sub>6</sub>-SUMO-R648 (right). **(B)** Purified megavirus tagged GciS sample at ~18 mg/ml in CAPS buffer at pH 10.5.



#### Fig S2: GciS oligomeric state.

(A) Gel filtration chromatography of mimivirus GciS. The absorbance at 280 nm is plotted in blue relative to the column volume in ml. The protein appears to be eluted close to the S200 26/60 column dead volume (~115 ml). The black curve corresponds to a calibration curve superimposed to the chromatogram and the different calibration proteins are numbered as follows 1: Dextran blue (~115 ml; 2,000,000 Da), 2: Aldolase (~191 ml; 158,000 Da), 3: Ovalbumin (~218 ml; 43,000 Da), 4: RNase A (~258 ml; 13,700 Da). (B) Negative staining of His<sub>6</sub>-SUMO tagged mimivirus GciS. Scale bar 100 nm.



**Fig S3**: (a) Mimivirus recombinant tagged (His<sub>6</sub>-SUMO) GciS purified aerobically and solubilized either in 10mM acetate at *pH* 4 (protein concentration ~ 27 mg/mL) or in 20 mM imidazole at *pH* 7.8 (protein concentration ~ 43 mg/mL). (b) Optical spectra of the recombinant protein solubilized at *pH* 4 and 7.8. (c) X-band EPR spectra of the recombinant protein solubilized at *pH* 4 (protein concentration of 0.23mM) and 7.8 (protein concentration of 0.54mM). The asterisk in panel c marks the position of the derivate EPR line near  $g \sim 4.3$  associated to nonspecific (adventitious) ferric iron. EPR conditions: Temperature, 15 K; microwave frequency, 9.481 GHz; modulation amplitude, 1 mT; microwave power, 10 mW.



**Fig S4**: Optical and X-band EPR spectra of megavirus recombinant tagged (His<sub>6</sub>-SUMO) GciS purified at *pH* 10.5 anaerobically (black trace) and after exposure to air (red trace). EPR recording conditions: Temperature, 15 K; microwave frequency, 9.481 GHz; modulation amplitude, 1 mT; microwave power, 10 mW. Protein concentration was 0.45 mM in EPR experiments.



**Fig S5**: Estimation of the *D* parameter from the temperature dependence of the g = 4.3 signal associated to the S = 5/2 linear  $[3Fe-4S]^{1+}$  cluster in mimivirus GciS. EPR spectra were recorded under non-saturating conditions between 3.6 and 40 *K*. Experimental data 1 and 2 were obtained from two mimivirus recombinant GciS samples, aerobically purified and solubilized either in acetate *pH* 4 (•) or CAPS *pH* 10.5 (•) buffers. Fits correspond to statistical Boltzmann distribution of the "*Ms* = ± 3/2" Kramers doublet population assuming a fully rhombic S = 5/2 system (*E*/*D* = 1/3) with absolute value of *D* varying between 0.2 cm<sup>-1</sup> (for  $\Delta/k_B = 1$  *K*) and 2 cm<sup>-1</sup> (for  $\Delta/k_B = 10$  *K*). The best fist was obtained for  $D = 0.6 \pm 0.2$  cm<sup>-1</sup> (black line).

#### Estimation of the D parameter:

The *D* parameter of the S = 5/2 linear  $[3Fe-4S]^{1+}$  species was estimated from the analysis of the temperature dependence of the g = 4.3 signal associated to the " $M_S = \pm 3/2$ " doublet. Briefly, the magnetic properties of the S = 5/2 linear  $[3Fe-4S]^{1+}$  species are described according to the spin Hamiltonian, which can be written as:

$$Hs = D\left(\boldsymbol{S}_{z}^{2} - \left(\frac{1}{3}\right)\boldsymbol{S}(\boldsymbol{S}+1)\right) + E\left(\boldsymbol{S}_{x}^{2} - \boldsymbol{S}_{y}^{2}\right) + \beta \boldsymbol{B}\boldsymbol{g}\boldsymbol{S}$$

The first two terms describe the zero-field splitting (ZFS), incorporating the axial (*D*) and rhombic (*E*) parameters at zero-field, while the last term describes the Zeeman interaction. The *E/D* parameter is commonly used to describe the rhombicity of the system. We assumed that the zero-field splitting term (~ *D*) was larger in energy than the Zeeman term (~ 0.3 cm<sup>-1</sup> at X-band), as it is often observed for transition metal complexes. In the absence of an external magnetic field, the *S* = 5/2 multiplet splits into three Kramers doublets " $M_S = \pm 1/2$ ", " $M_S = \pm 3/2$ " and " $M_S = 5/2$ ", which are actually mixed states of the pure  $M_S$  states as a function of *D*, *E*. The energy gaps  $\Delta_1$ , between the  $\pm 1/2$  and  $\pm 3/2$  doublets, and  $\Delta_2$ , between the  $\pm 3/2$  and  $\pm 5/2$  doublets, rely on the ZFS parameters. For an axial system (*E/D* = 0),  $\Delta_1$  and  $\Delta_2$  correspond to 2*D* and 4*D*, respectively. For a fully rhombic system (*E/D* = 1/3),  $\Delta_1$  and  $\Delta_2$  are equal and correspond to ~ 3.5*D*. <sup>1-2</sup>

The position of the resonance lines on the X-band spectrum (**Fig. 2**) indicated that the S = 5/2 center was almost fully rhombic, with  $E/D \sim 0.27$ . We thus made the convenient assumption of a fully rhombic (E/D = 1/3) zero-field splitting case, since in this case the Boltzmann distribution of the " $M_S = \pm 3/2$ " middle doublet depends only on the absolute value of the D term, thus leading to **Eq. 1**:

$$N_{\pm 3/2} = \frac{N}{1 + e^{\frac{3.5D}{k_B T}} + e^{\frac{-3.5D}{k_B T}}} \propto I.T$$
 (Eq. 1)

where  $N_{\pm 3/2}$  and N correspond to the populations of the  $M_s = \pm 3/2$  sub-level and of the S = 5/2 manifold, respectively,  $k_B$  is the Boltzmann constant (~ 0.7 cm<sup>-1</sup> K<sup>-1</sup>). The  $N_{\pm 3/2}$  population is proportional to the intensity of the  $g_{\text{eff}} \propto 4.3$  line over the temperature (**Eq. 1**) and can therefore be experimentally estimated.



**Fig S6:** Zoom on the 100 to 99.3–99.0% transmission part of the experimental Mössbauer spectra of mimivirus GciS (**Fig. 4 in the manuscript**). Spectra were recorded at *ca* 6 K using an external magnetic field parallel to the  $\gamma$ -beam of 0.06, 4, and 7 T. The overlaid red solid lines are the sub-spectra of the linear *S*=5/2 ground state [3Fe-4S]<sup>1+</sup> cluster. Contributions of the three ferric iron sites are shown above (green, orange, and purple lines).

Mimivirus	MS <mark>C</mark> FGGWNGGCGPCGGFGWGGCGPSWGCGPCGFGGGYSYRVSYGFGGCGFFGGFGWGGCC	Clade A: 9 Cys
Moumouvirus	MS <mark>C</mark> GP <mark>C</mark> GGFGLGGFGGISGYAYGFSYNNNFGG <mark>C</mark> GPFGGYYRG <mark>C</mark> GPNWG <mark>C</mark> NP <mark>C</mark> GGWGWGGW <mark>C</mark>	
Moumouvirus australiensis	MS <mark>C</mark> GP <mark>C</mark> GFGGLGFGGGYGAVSGYAYGFNYGTGFGGG <mark>C</mark> GPWGGYYRPGWGA <mark>C</mark> NP <mark>C</mark> GGWGWGNGW <mark>C</mark>	Clade B: 5 to 7 Cys
Moumouvirus maliensis	MS <mark>C</mark> GP <mark>C</mark> GFGGGFGGISGYAYGFNYNTGFGGG <mark>C</mark> GPFYGGYYRPGWGG <mark>C</mark> NP <mark>C</mark> GGWGWGGW <mark>C</mark>	
Megavirus chilensis	MS <mark>C</mark> GIGGFGG <mark>C</mark> GP <b>C</b> GFGGGFGGISGYAYGFNYGTNFG <mark>C</mark> GPWGGYYRG <mark>C</mark> SP <mark>C</mark> GWGNGWGGWGGGGG <mark>CC</mark>	Clade C: 8 Cys
Tupanvirus soda lake	MS <mark>C</mark> YRGP <mark>C</mark> GGYGWGYPGFGYGSG <mark>CC</mark> YPRYNA <mark>C</mark> GYGGFSGFGGYGYNYGYARGGSWGWGW	Clade D: 5 Cys

**Fig S7:** GciS protein sequences for each viral clade prototype (A-D). Three representative sequences are shown for the most variable B-clade. Cysteines are highlighted in yellow.

**Table S1**. Estimation of iron and Fe-S cluster contents in recombinant air-purified GciS proteins from*Megavirinae* clades A-D.

Clade	GciS	Fe/GciS <sup>a</sup>	A420nm/A280nm <sup>b</sup>	[3Fe-4S] <sup>1+</sup> /GciS <sup>c</sup>	[2Fe-2S] <sup>2+</sup> /GciS <sup>d</sup>
Α	Mimivirus	$0.65\pm0.10$	0.12	0.10	0.18
В	Moumouvirus	$0.40\pm0.05$	0.09	0.07	0.10
	Moumouvirus australiensis	$0.21\pm0.05$	0.06	0.04	0.05
	Moumouvirus Maliensis	$\textbf{0.27} \pm \textbf{0.05}$	0.07	0.05	0.06
C	Megavirus chilensis	$0.92\pm0.10$	0.11	0.12	0.28
D	Tupanvirus soda lake	$0.44\pm0.05$	0.07	0.08	0.10

<sup>a</sup> The total iron content per protein was determined either by ICP-MS and/or Fish method.

<sup>b</sup> The absorbance ratio was determined from the UV/vis spectra presented on **Fig. 5**.

<sup>c</sup> The (*S*=5/2) linear [3Fe-4S]<sup>1+</sup> content per protein was estimated from spin quantification of the  $g \sim 4.3$  signal shown on **Fig. 5**. Error uncertainty on the linear [3Fe-4S]<sup>1+</sup> is about 30% of the value given in **Table S1**.

<sup>d</sup> The [2Fe-2S]<sup>2+</sup> content per protein *was calculated* from the total iron content and spin intensity determination for the linear [3Fe-4S]<sup>1+</sup> as follows:

[2Fe-2S]/protein = 1/2 x (total iron/protein - 3x[3Fe-4S]/protein)

### **Materials and methods**

#### Genes cloning and protein production

The *R633b* gene was amplified from *mimivirus* genomic DNA. The PCR product was inserted by In-Fusion HD cloning kit (Takara) into an in-house-modified pETDuet expression vector (Novagen), between the *BamHI/NotI* restriction sites, allowing the production of the protein fused with a cleavable N-terminal His<sub>6</sub>-SUMO tag. A human rhinovirus 3C (Prescission) protease cleavage site allowed tag removal. The cloning was controlled by sequencing (Eurofins).

The genes encoding the Mg762, mm\_612b, and ma671a proteins were amplified from *megavirus chiliensis, moumouvirus maliensis, and moumouvirus australiensis genomic DNA* respectively. The genes encoding the tupanvirus soda lake QKU35266 and moumouvirus (unannotated gene, Genbank ID: JX962719.1, sequence regions 770054 to 770236) proteins were synthetically made (GenScript). They were then cloned as described for *R633b*.

The vectors were transformed in *E. coli* Rosetta (DE3). Cells were grown in 2YT medium containing 100  $\mu$ g/mL ampicillin and 34  $\mu$ g/mL chloramphenicol at 37 °C to an  $A_{600}$  of ~0.7 when the culture temperature was shifted to 17 °C. Protein production was induced at  $A_{600}$  ~1, by the addition of 0.5 mM IPTG. Cells were grown 16 h after induction and harvested by centrifugation at 6000 *g* for 15 min at room temperature.

#### <sup>57</sup>Fe-enriched protein production

To produce the <sup>57</sup>Fe-labeled protein sample, the transformed bacteria were grown as described above in a different medium using a homemade minimum medium containing 40 mM MOPS *pH* 8.3, 4.5 mM Tricine, 25 mM NaCl, 40 mM KOH, 0.5 mM MgCl<sub>2</sub>, 10  $\mu$ M Mohr's salt (NH<sub>4</sub>)<sub>2</sub><sup>57</sup>Fe(SO<sub>4</sub>)<sub>2</sub>.6H<sub>2</sub>O, 0.5  $\mu$ M CaCl<sub>2</sub>, 0.4  $\mu$ M H<sub>3</sub>BO<sub>3</sub>, 0.08  $\mu$ M MnCl<sub>2</sub>, 0.03  $\mu$ M CoCl<sub>2</sub>, 12 nM CuCl<sub>2</sub>, 1  $\mu$ M ZnCl<sub>2</sub>, 1.2  $\mu$ M Na<sub>2</sub>MoO<sub>4</sub>, 50 mM KPO<sub>4</sub> *pH* 7.5, 275 nM K<sub>2</sub>SO<sub>4</sub>, 0.2% Glucose, 1x Mem Vitamins (Thermo Fisher) and 10  $\mu$ M NH<sub>4</sub>Cl. The Mohr's salt synthesis was done according to the protocol described in ref.<sup>3</sup>

#### **Protein purification**

All purification steps were performed in aerobia, unless otherwise mentioned. For each produced protein the purification was made in two steps with the appropriate resuspension buffer. Bacteria pellets were resuspended in buffer A (50 mM Tris-HCl, 300 mM NaCl, *pH* 8.5 for R633b and Mg762 proteins, and 50 mM Tris-HCl, 150 mM NaCl, *pH* 9.0, for AUL79376.1, mm\_612b, ma671a, and moumouvirus proteins, containing 0.1 mg/mL DNase, 0.1 mg/mL lysozyme, 0.2% Triton X-100 and antiprotease tablets (Roche Applied Science). Cells were lysed by 6 cycles of 3 min sonication. The lysate was clarified by centrifugation at 13,000 *g* for 45 min at room temperature (RT) and filtrated through a 0.2 µm PES filter (Millipore) before being loaded on Ni-NTA resin (Thermo Scientific) pre-equilibrated with buffer A. The column was washed with 10 column volumes of buffer A and 10 column volumes of buffer A containing 50 mM imidazole. Elution was performed by steps of 5 column volumes at 0.25, 0.5, and 1M imidazole. The protein was eluted during the 0.25M step. All fractions were controlled by SDS-PAGE electrophoresis. All the purification steps were performed at room temperature (RT), as the protein did precipitate under 10°C.

Microdialysis tests were performed using different buffers, which returned that the protein was soluble in CAPS *pH* 10.5 buffer. Thus, the purified tagged protein (~20 kDa) was dialyzed on a 12.5 kDa cut-off, regenerated cellulose membrane (Spectrum), in 50 mM CAPS *pH* 10.5 for 16h at RT, and concentrated on a 30 kDa concentrator (GE Healthcare) at RT.

Concentrations of purified GciS (Glycine, cysteine-rich Fe-S) were between 100  $\mu$ M (~2 mg/mL) and 3 mM (~60 mg/mL) for EPR experiments, and up to 7 mM for Mössbauer experiments. The purified GciS protein formed a gel when concentrated at 7 mM and can be reversibly solubilized by temperature increase.

#### Gel filtration chromatography

Analytic gel filtrations were performed to estimate the oligomeric state of the proteins on an ÄKTA Explorer FPLC (GE Healthcare), using a S200 26/60 Gel filtration column (GE Healthcare), pre-equilibrated with buffer 50 mM CAPS, 150 mM NaCl, *pH* 10.5.

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#### Negative staining electron microscopy

Negative staining grids were prepared by adsorbing a 7 µl sample drop at 50 µg/ml or 250 µg/ml, in a 300 mesh ultra-thin carbon-coated copper grid (Electron Microscopy Sciences, EMS) for 3 min, washed with two drops of filtered ultra-pure water, and stained 2 min in 2% uranyl acetate. Grids were then observed on a FEI Tecnai G2 200 kV microscope, with an Olympus Veleta 2k. The different measurements were performed using ImageJ 1.52i.<sup>4</sup>

#### Protein concentration estimation

Estimation of GciS protein concentration was routinely achieved by measurement of the absorbance at 280 nm using a calculated extinction coefficient derived from the primary sequence of the SUMOtagged protein (Protparam, Expasy):  $\varepsilon_{mimivirus} = 28460 \text{ M}^{-1} \text{ cm}^{-1}$ ,  $\varepsilon_{megavirus} = 31440 \text{ M}^{-1} \text{ cm}^{-1}$ ,  $\varepsilon_{moumouvirus} = 31315 \text{ M}^{-1} \text{ cm}^{-1}$ ,  $\varepsilon_{australiensis} = 38305 \text{ M}^{-1} \text{ cm}^{-1}$ ,  $\varepsilon_{maliensis} = 32805 \text{ M}^{-1} \text{ cm}^{-1}$  and  $\varepsilon_{tupan} = 40130 \text{ M}^{-1} \text{ cm}^{-1}$ . For megavirus GciS, we could provide a corrected extinction coefficient value ( $\varepsilon_{280nm}$  of 29 700 (±700) M<sup>-1</sup> cm<sup>-1</sup>) according to quantification of aminoacids after hydrolysis (Service Platform, Chemistry of Biomolecules, Dr Françoise Baleux, Institut Pasteur).

#### Metal content analysis and acid-labile sulfide determination

Metal content in recombinant purified GciS proteins was measured by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, ICAPQ, Thermo Electron, equipped with a collision cell). When possible, measurements were done in triplicate on 400 μL samples with a protein concentration of 10 μM. Before analysis, samples were treated with nitric acid (Purissime, Sigma Aldrich), heated at 70°C and diluted with ultrapure water. Calibration was done using external certified standards (Multi-elements, SCP Science). The presence of various transition metal in the samples were investigated (iron, zinc, nickel, manganese, copper, chromium, cobalt, molybdenum, cadmium, scandium, titanium); only iron and occasionally traces of zinc (<5% of the protein concentration) were detected. Iron content determination was determined either by ICP-MS or according to the Fish colorimetric method provided by Dr S. Ollagnier CEA Grenoble, and described in ref <sup>5</sup>. Inorganic sulfide determination was achieved according to the initial publication of Beinert<sup>6</sup> and using the adapted protocol provided by Dr S. Ollagnier and described in ref <sup>5</sup>.

#### Preparation of whole Ec cells and purified mimivirus particles for EPR experiments

For EPR spectroscopy experiments on whole bacterial cells, the *R633b* gene was inserted using the Gateway system into a pDEST17 expression vector, allowing the production of protein fused with a non-cleavable N-terminal His<sub>6</sub>-tag.

The vector was transformed in *E. coli* C41 (Novagen). Cells were grown in SB medium containing 100  $\mu$ g/mL ampicillin at 37 °C. At  $A_{600} \sim 0.9$  the culture was shifted to 17 °C. Protein production was induced at  $A_{600} \sim 1.3$ , by the addition of 1.3 mM IPTG. Cells were grown 16 h after induction and harvested by centrifugation at 6000 *g* 15 min at room temperature.

To produce the mimivirus particles, three T175 flasks of *Acanthamoeba castellanii* cultures were infected, once the cells were lysed, the culture medium was centrifuged at 500 g for 10 min to remove cell debris and the supernatant was centrifuged at 6800 g for 45 min, then washed in K36.<sup>7</sup> Mimivirus particles were purified according to ref.<sup>7</sup> The viral production was controlled on a Zeiss microscope.

Pellets obtained from viral particles (5x10<sup>11</sup>), or the bacterial pellets, were washed three times with deionized water then centrifuged in order to remove undesired paramagnetic contaminants (such as adventitious Mn<sup>2+</sup> or Fe<sup>2+</sup> ions) from the culture medium. We also used Tris buffer (40mM Tris-HCl, *pH* 8.5) containing 1mM EDTA as a washing solution for chelating metal contaminants, but found no difference in the background EPR spectra.

#### Spectroscopic experiments

UV-Visible absorption spectra were recorded on dual-mode Lambda-25 Perkin-Elmer or Safas spectrophotometers using a 1 mm pathlength quartz cuvette (Hellma). Protein concentration for mimivirus GciS was 300  $\mu$ M in 50 mM CAPS buffer *pH* 10.5 (**Fig. 2a**).

EPR spectroscopy experiments were performed on a Bruker Elexsys E500 spectrometer fitted with an Oxford Instruments ESR-900 helium-gas flow for cryogenic temperature studies. Spin number determination of the S=5/2 species was achieved using home-calibrated quartz fused EPR tubes and an external S=5/2 Fe(III) standard of known concentration (1mM Fe(III) dissolved in a 10mM EDTA,

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50mM Tris-HCl solution buffered at *pH* 8 with 10% glycerol). For that purpose, spectra were recorded at 15K and under non-saturating conditions (*P*=1mW at 15 K). We ensured that, at this temperature, the population of the  $M_s = \pm 3/2$  sublevel giving the *g*=4.3 signal reached a plateau for both the sample and Fe(III) standard (see **Fig. S3** for mimivirus GciS), representing one third of total spin population in accordance to the Boltzmann's distribution for a *S* = 5/2 manifold. Furthermore, *S* = 5/2 [FeEDTA]<sup>-</sup> complex has  $D \sim 0.7$  cm<sup>-1</sup> (and *E/D* = 0.328) similar to that of GciS protein, it is thereby an appropriate spin concentration standard.<sup>8</sup>

The number of spin S = 5/2 in the protein sample was then determined from the comparison of the double integration of the g = 4.3 peak for both spectra (protein sample and standard), and by considering the experimental recording parameters, EPR tube calibers and sample concentrations. Experimental recording parameters for EPR spectra: (**Fig. 2b**) temperature, 15 K; microwave frequency, 9.481 GHz; modulation amplitude, 1 mT; microwave power, 1 mW; (**Figs. 4b and 4c**) temperature, 15 K; microwave frequency, 9.481 GHz; modulation amplitude, 2 mT; microwave power, 10 mW. All EPR spectra shown in **Figs. 2 and 4** were normalized to the g=4.3 signal amplitude.

The Mössbauer spectra were recorded at ca. 5 K on a strong-field Mössbauer spectrometer equipped with an Oxford Instruments Spectromag 4000 cryostat containing an 8 T split-pair superconducting magnet. The spectrometer was operated in a constant acceleration mode in transmission geometry. The isomer shifts were referenced against that of a room-temperature metallic iron foil. Analysis of the data was performed with a home-made program.<sup>9-10</sup>

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