Workshop on Deep Underground Laboratory
Integrating Activity in biology (DULIA-bio)
Canfranc, October 13-14 2015

The underground biology at the Gran Sasso National Laboratory: from Pulex to Cosmic Silence

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on behalf of the CS collaboration

Istituto Superiore di Sanità

L'Aquila University

INFN Roma1-Gr. Coll Sanità

INFN-LNGS

INFN LNF

La Sapienza University, Rome

Flinders University, Adelaide, Australia

Environmental radiation represents a constant daily stimulus that has been incorporated in the biology of living organisms during evolution, with the development of defence mechanisms well preserved during phylogeny

In order to investigate if modulation of radiation environment can modify the biochemistry of biological systems and their response to genotoxic agents, Satta et al. (Mutat Res 1995) designed an experiment consisting in twin set-up of yeast culture in a laboratory where the environmental radiation is reduced as possible and in a reference laboratory

To this purpose they took advantage of the opportunity represented by the Gran Sasso underground laboratory of the Italian National Institute of Nuclear Physics (INFN)

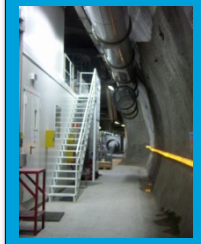
Experimental Approach

SET UP OF PARALLEL EXPERIMENTS UNDER DIFFERENT RADIATION ENVIRONMENTS

Low Radiation Environnment (LRE)

presence of 5 cm Fe shielding around the cell culture incubator

LNGS-INFN (underground lab)

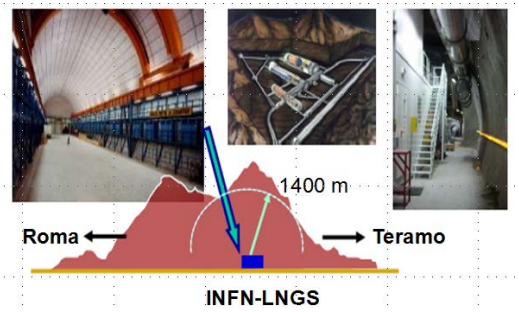


ISS / *L'Aquila Univ* (external labs)

Reference Radiation Environment(s) (RRE)

From the LNGS web site:

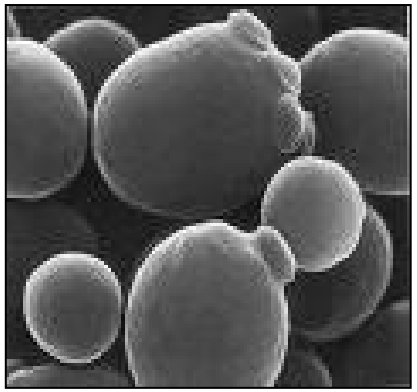
- The average 1400 m rock coverage gives a reduction factor of one million in the cosmic ray flux moreover, the neutron flux is thousand times less than on the surface, thanks to the smallness of the Uranium and Thorium content of the dolomite rocks of the mountain



- *The mission of the Laboratory is to host experiments that require a low background environment in the field of astroparticle physics and nuclear astrophysics and other disciplines that can profit of its characteristics and of its infrastructures*

(http://www.lngs.infn.it/lngs_infn/index.htm?mainRecord=http://www.lngs.infn.it/lngs_infn/contents/lngs_en/public/about/)

Pioneering work of Satta et al.



Reference Laboratory: Institute of Genetics, "La Sapienza" University, Rome

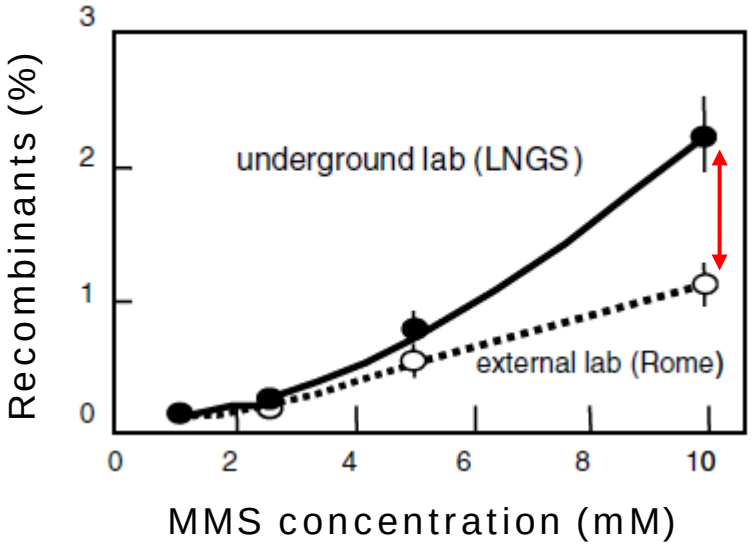
Cell line (yeasts): Saccaromyces cerevisiae

Culture time: 1week (120 generations)

Genotoxic agent: Methyl methan sulphonate (MMS), radiomimetic compound

Results:

Higher frequency of recombination in yeast cells grown underground LNGS, respect to those grown at La Sapienza University (Rome)



Satta et al., Mut Res,1995

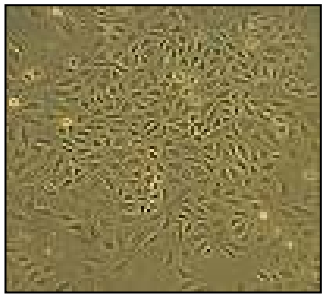
From yeasts to mammalian cells

After this first experiment a collaboration started with the ISS and a cell culture laboratory was set up underground the LNGS

Studies have been carried out on cells from higher eukaryotes cultured for several months, in order to reach a comparable number of generations as yeasts, in low and in reference radiation environments

The PULEX experiment

Reference Laboratory: Istituto Superiore di Sanità (ISS), Rome

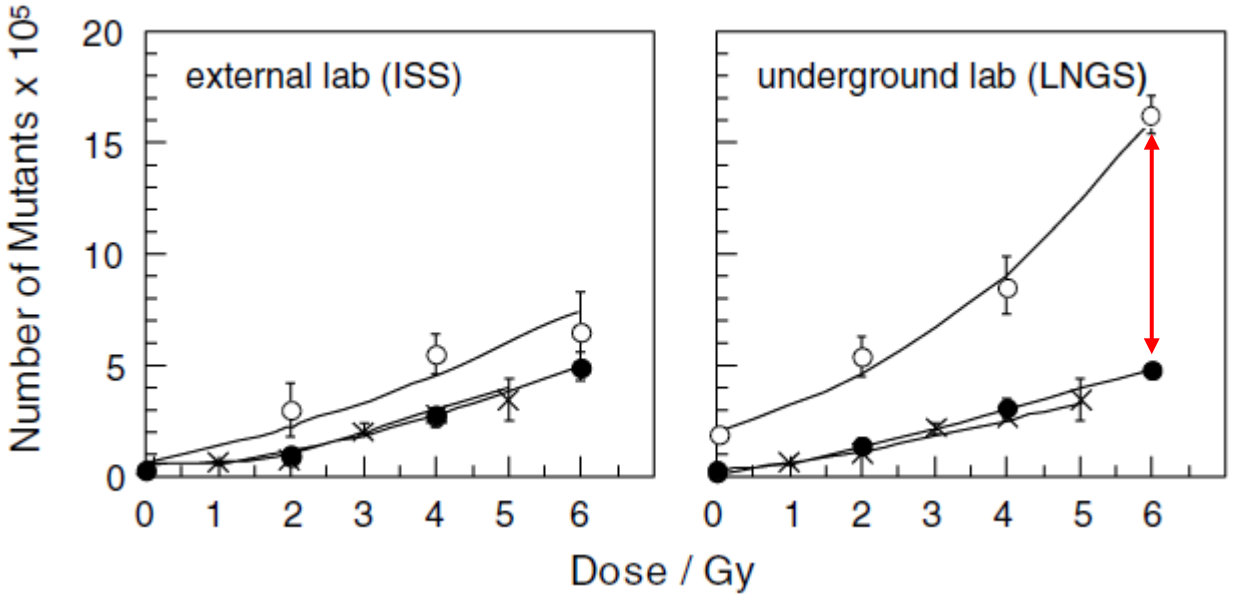


Mammalian cell line (rodent): V79 Chinese hamster lung fibroblasts

Culture time: 3 and 9 months Genotoxic agent: X-rays

Black: 3 months

White: 9 months

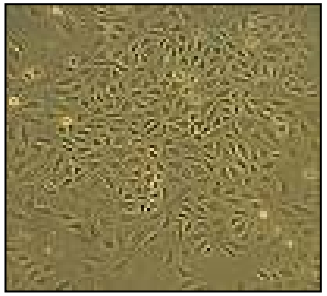


Results: Higher mutation frequency in cells after 9 months of growth in reduced radiation environment

Satta et al., Rad Environ Biophys, 2002; Antonelli et al., Il Nuovo Cimento, 2008

The PULEX-2 experiment

Reference Laboratory: LNGS external lab.

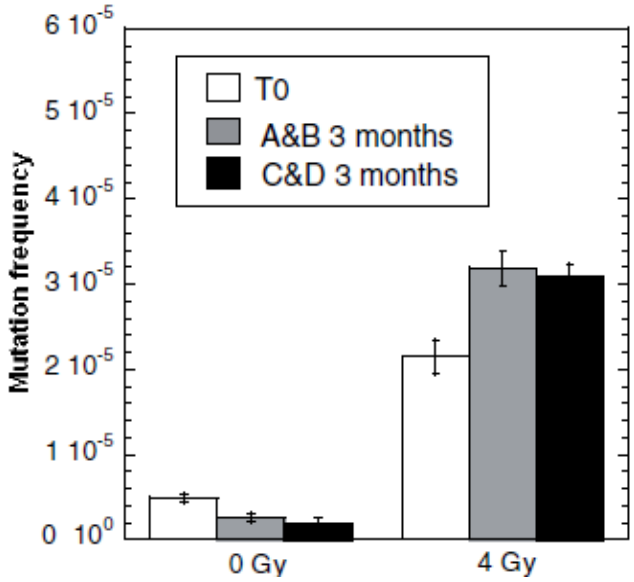


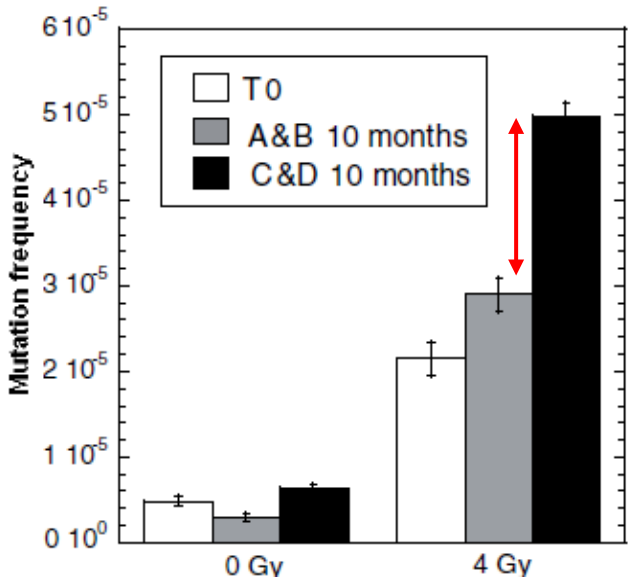
Mammalian cell line (rodent): V79 Chinese hamster lung fibroblasts

Culture time: 3 and 10 months; Genotoxic agent: X-rays

A&B: external cultures

C&D: underground cultures

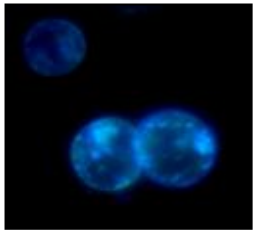




Results: Higher mutation frequency in cells after 10 months of growth in reduced radiation environment

Satta et al., Rad Environ Biophys, 2002; Antonelli et al., Il Nuovo Cimento, 2008

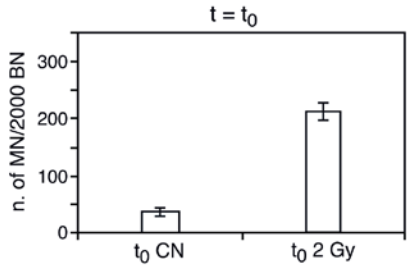
The COSMIC SILENCE experiment



Reference Laboratory: Istituto Superiore di Sanità (ISS), Rome

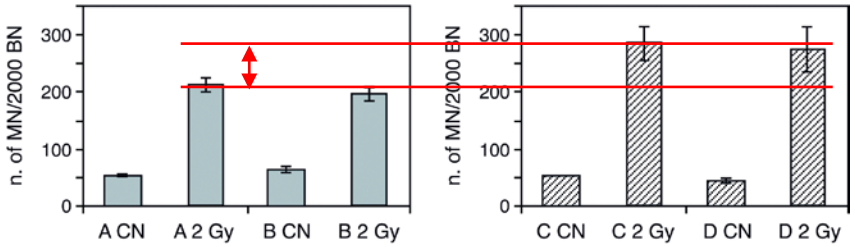
Mammalian cell line (human): TK6 Lymphoblasts

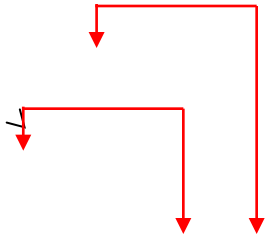
Culture time: 6 months; Genotoxic agent: X-rays



ISS
t = 6 months of culture

LNGS
t = 6 months of culture





Cn 1Gy Cn 1Gy

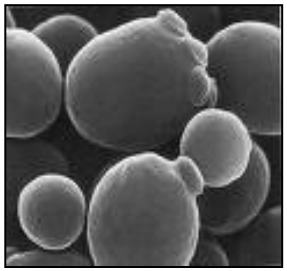
Results: Higher micronuclei induction and reduced capability of ROS scavenging in cells grown in reduced radiation environment

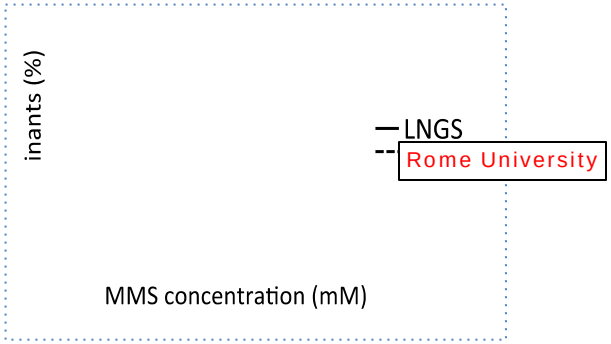
Carbone et al., Rad Environ Biophys, 2009

Carbone & Pinto et al., Il Nuovo Cimento, 2010

Long term experiments on in vitro models

Satta et al., 1995





S. Cerevisiae

120 generations – 1 week

Satta et al., 2002
Antonelli et al., 2008
Fratini et al., 2015

Chinese hamster cells

Pulex

ISS

Dose (Gy)

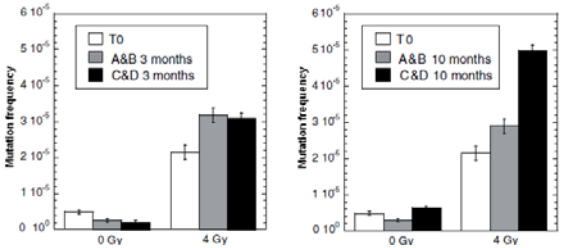
9 months of culture

Cells cultured in reduced environmental radiation conditions for several months are:

- *Less tolerant to radiation-induced DNA damage*
- *Less efficient in scavenging reactive oxygen species*

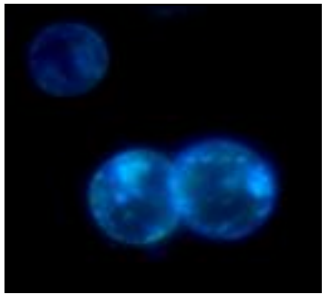
Pulex-2

Similar results at LNGS external as reference lab



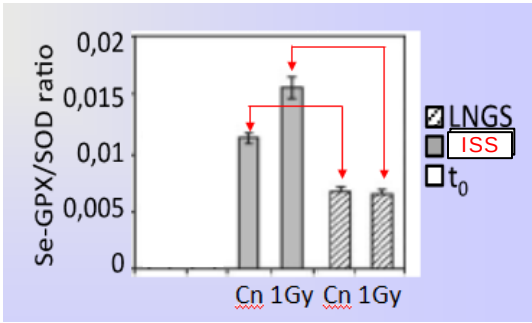
A&B: external cultures
C&D: underground cultures

Carbone et al., 2009
Carbone & Pinto et al., 2010



Human cells

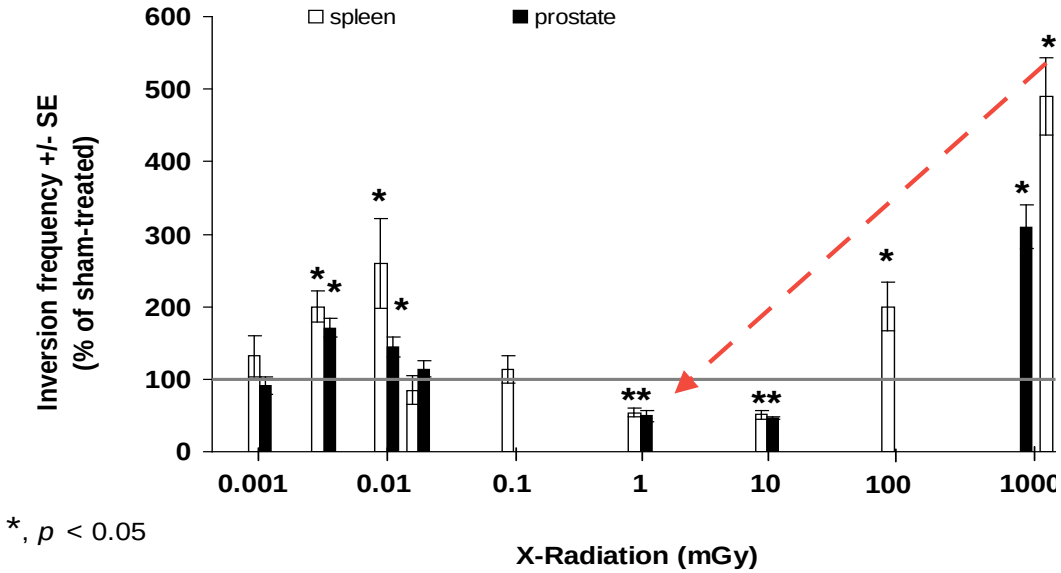
Cosmic Silence



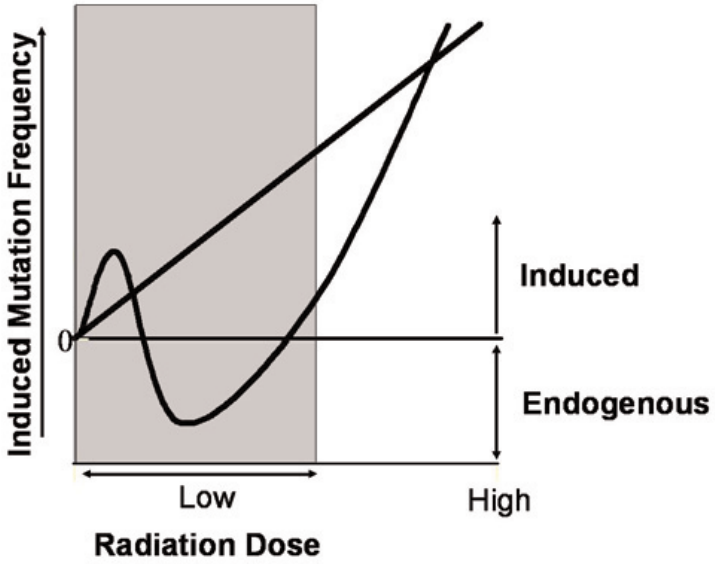
6 months of continuous culture

The collaboration with the Flinders University

pKZ1 response to acute-dose of ionizing radiations



Zeng et al., 2006



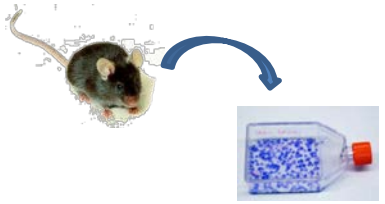
Sykes et al., 2006

Low dose radiation dose-response curve. Inversions were induced in pKZ1 at very low and at high doses of radiation exposure. Intermediate doses of radiation caused a decrease below endogenous inversion frequency.
The straight line represents the LNT theory.

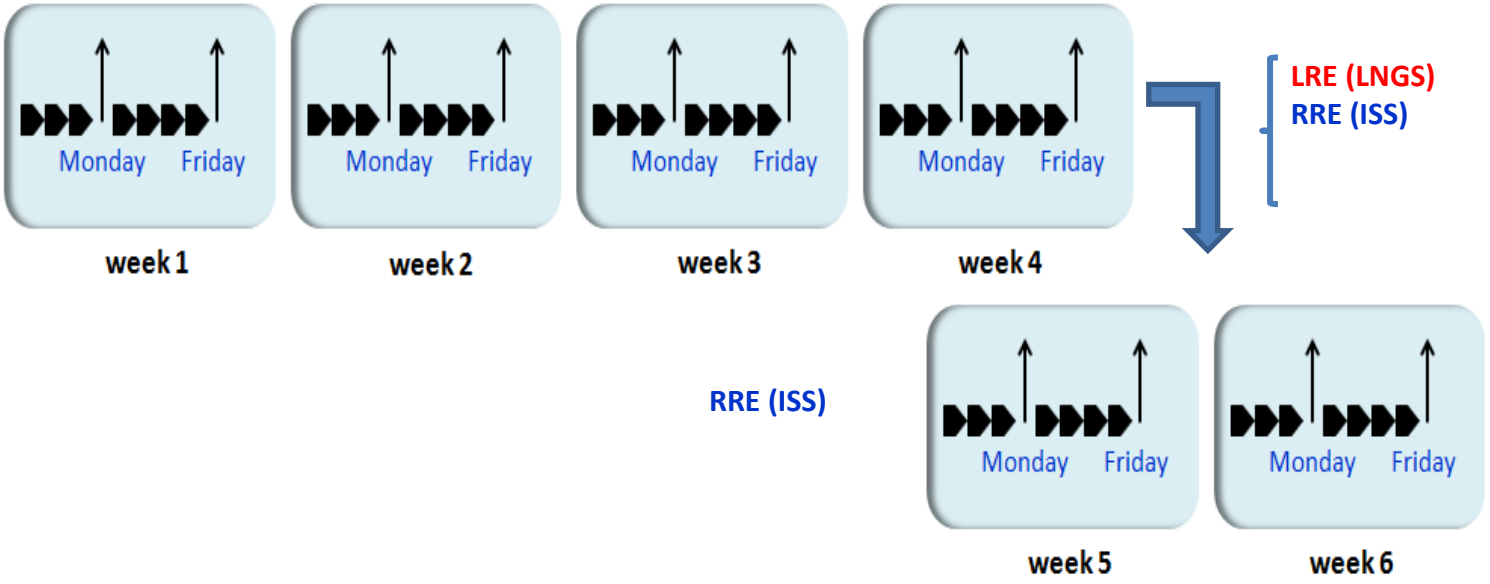
The COSMIC SILENCE short term experiments

Reference Laboratory: Istituto Superiore di Sanità (ISS), Rome

Mammalian cell line (rodent): A11 cells isolated from pKZ1 mouse, kindly donated by Prof. P.Sykes (Flinders University, Adelaide, Australia)



Culture time: up to 1 month in RRE and LRE

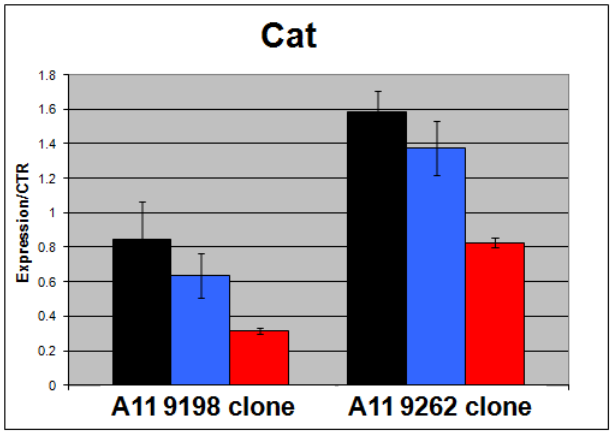


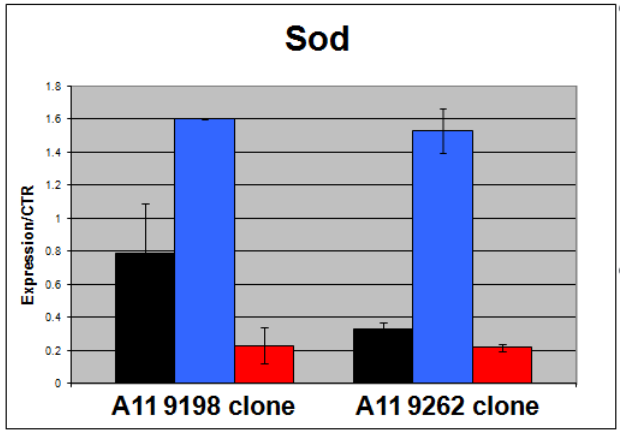
Expression of genes involved in the protection from oxidative damage

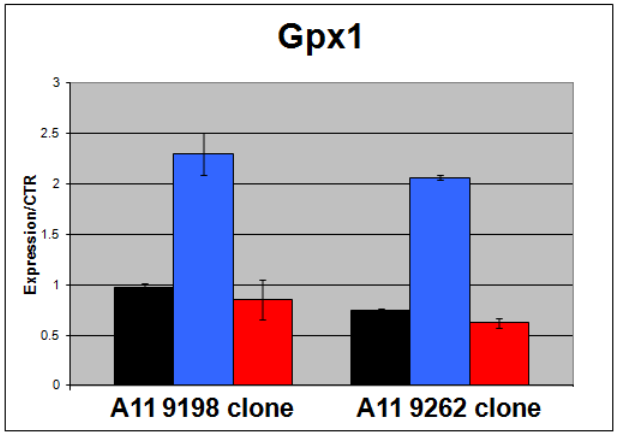
time 0

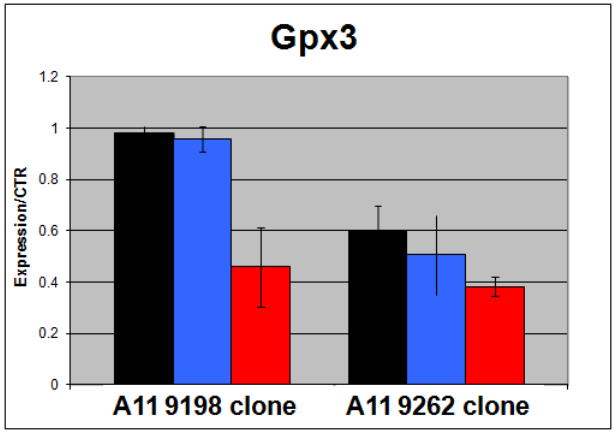
1 month - 4 parallel cultures at RRE (ISS)

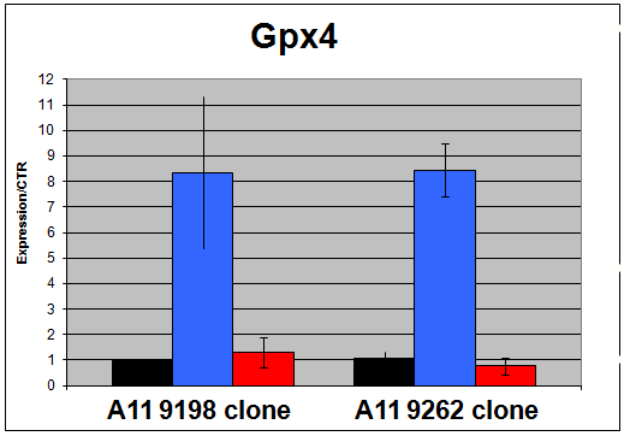
1 month - 4 parallel cultures at LRE (LNGS)

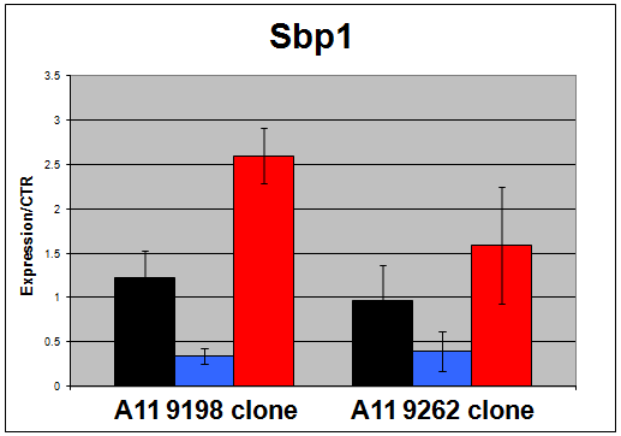






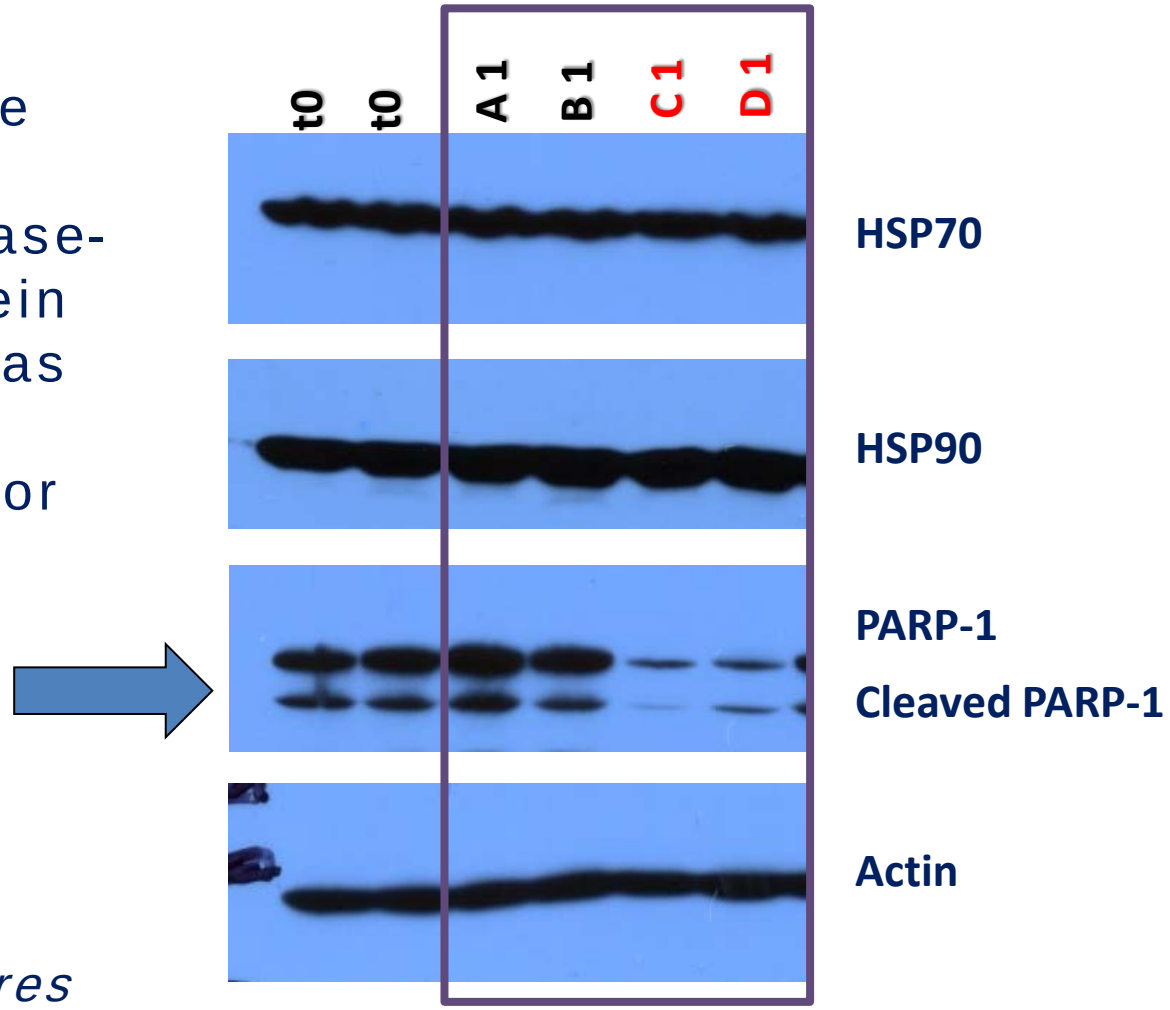






Quantitative protein analysis: Parp-1

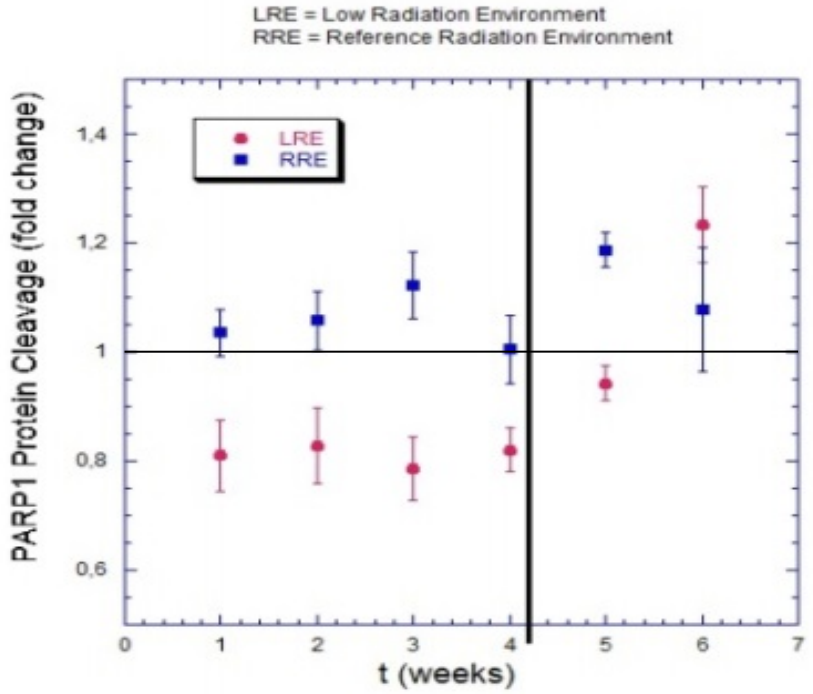
After 1 month of continuous culture the concentration of poly (ADP-ribose) polymerase-1 (Parp-1), a key protein in DNA repair as well as in differentiation, proliferation, and tumor transformation, is drastically reduced in cells grown in LRE

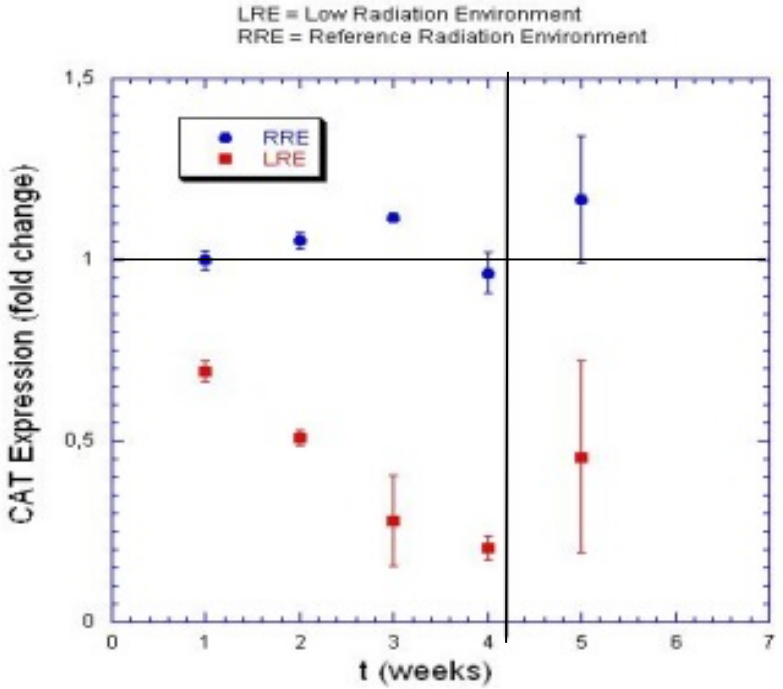


A&B: ISS external cultures
C&D: LNGS underground cultures

Experiments carried out on pKZ1 A11 cells cultured for 1 month only (instead of several months) in both LRE and RRE confirmed that extremely low and protracted doses, as those comparable to the radiation environment, are capable to modify the metabolisms and the stress response capability of biological systems. Moreover,

- when cells grown in LRE for 1 month are subsequently taken and cultured in RRE, the cleavage of PARP-1 is restored
- a similar trend is observed for the stress response genes

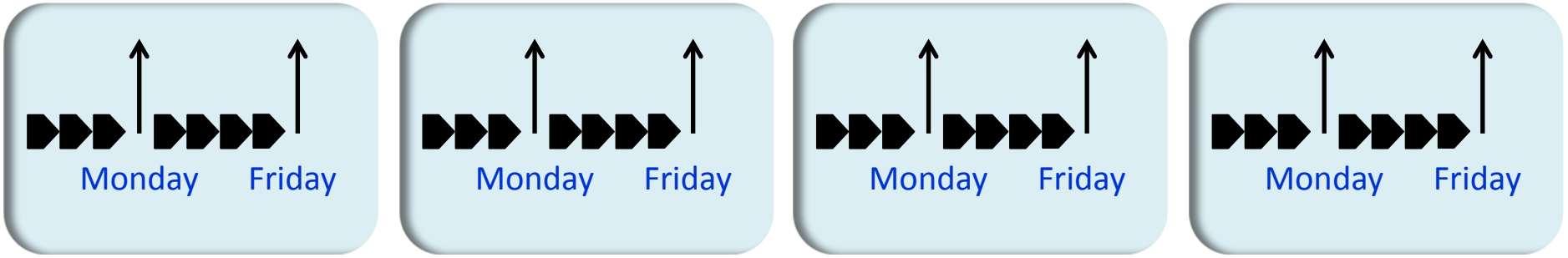




Cosmic Silence:
recent results on A11 cell line

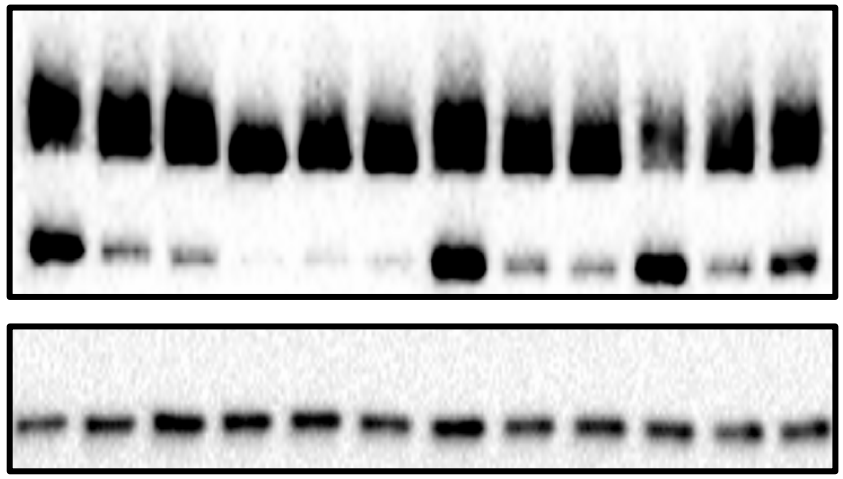
Modulation of gamma dose by the presence or absence of Fe-shield in LRE: measurements on the expression levels of PARP-1

Cleavage of PARP-1 protein has been studied in A11 cells grown for 4 weeks in 3 different environmental radiation conditions: RRE at the ISS; LRE at the LNGS in the presence or absence of Fe shield



week 1 **week 2** **week 3** **week 4**

- ***PARP-1 cleavage start after the 3rd day of exponential growth***
- **At the 4th day of culture: LRE cells show a significantly lower level of PARP-1 cleavage than RRE cells**
- **The presence of Fe shield does not affect the LRE cell response**



OUT = RRE (ISS) **IN-S = LRE (LNGS with Fe shield)** **IN = LRE (LNGS withot Fe shield)**

1-3 and 4w samples have been collected after 4 days of exponential culture; the 2w samples have been collected after 3 days of exponential culture (no PARP-1 cleavage is expected)

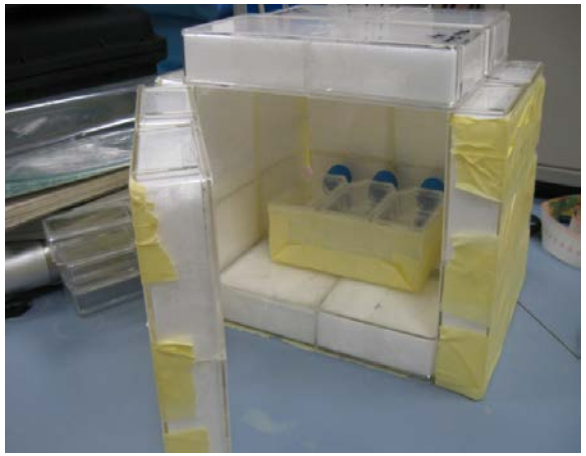
Preliminary experiments on gene expression conducted on cells grown at LRE did not show difference between cells grown in the presence or absence of 5 cm Fe shield *(able to reduce the gamma component of the radiation spectrum by a factor of about 10)*

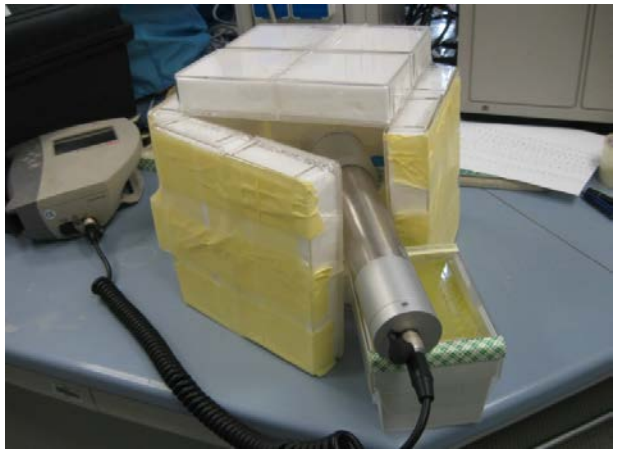
This finding indicates that a 10 fold increase in the gamma component increase of the environmental radiation does not significantly influence the biological response

In the attempt to expose the cells growing in LRE to known low doses of ionizing radiation experiments started in collaboration with J.B. Smith (Mexico State University) aimed at increasing the background at the LRE using KCL salt as radiation source

V79 Chinese hamster cells have been cultured at LRE in different radiation conditions, namely in the presence of shielding or in the presence of KCl salt quantity able to increase the background level up to about 50 nGy/h (being about 3.6 nGy/h in the shielded incubator). Cell growth and gene expression have been investigated. Data analysis is in progress

Measurements carried out with the help of Matthias Laubenstein and Giuseppe Di Carlo

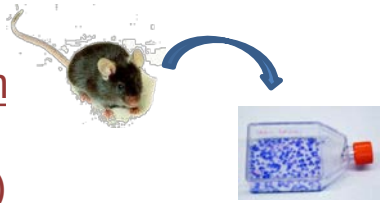




Short term experiments on in vitro models

Cosmic Silence

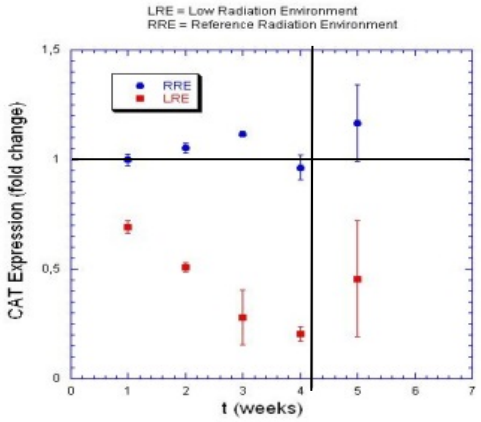
A11 cells derived from the pKZ1 mouse (radiosentivive model)



week 1 | week 2 | week 3 | week 4 — Monday … Friday

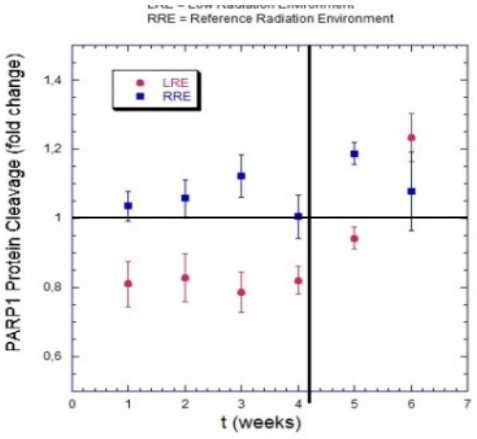
LRE (LNGS)
RRE (ISS)

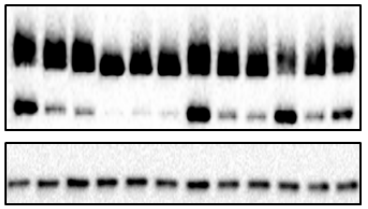
RRE (ISS) — week 5 | week 6 — Monday … Friday



Antioxidant enzymes (e.g., catalasi)

PARP-1





weeks 1w 1w 1w 2w 2w 2w 3w 3w 3w 4w 4w 4w

out in-s in out in-s in out In-s in out in-s in

OUT = ISS

IN-S = LNGS with Fe shield
IN = LNGS withot Fe shield

Fratini et al., MELODI Workshop 2014, RRS 2014, ICRR 2015

After 4 weeks of culture in different radiation environment:

- *Divergencies are observed in the expression of enzymed with anti-oxidant activity*
- *Activation of PARP-1, a key protein in DNA damage repair, apoptosis, proliferation ..., is reduced in LRE conditions, indipendently of the presence of a shielding able to reduce the gamma component by a factor of about 10*
- *Such divergencies are reduced when LRE cells are subsequently cultured in RRE*

Crucial issue

Which are the components of the radiation spectrum major responsible for the biological differences observed between the underground and the external radiation environments ?

Inside the Gran Sasso mountain the radiation environment is composed essentially of low energy γ-rays of local origin (low-LET radiation), whose spectrum extends to about 3 MeV

The results until now obtained on in vitro models suggest a scarce influence of the gamma component

In principle, the thickness and the sedimentary origin of the overburden makes negligible the contribution of cosmic rays and of neutrons (Rindi et al. 1988)

A new characterization of the radiation field in the different experimental sites is ongoing

Accurate measurements of the neutron component using BF3 detectors (in horizontal and vertical position at 150 cm from the floor, Surrounded by 1.5 mm of Cd (*Cadmium cut-off=0.5 eV*) or by 12.5 cm polyethylene)

Gamma spettroscopy with HpGe

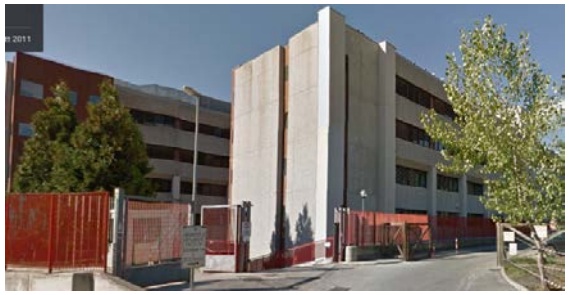
Dosimetric measurement using TLD 700H and high pressure ionization chamber

Radon monitoring in aria using Alfaguard equipment

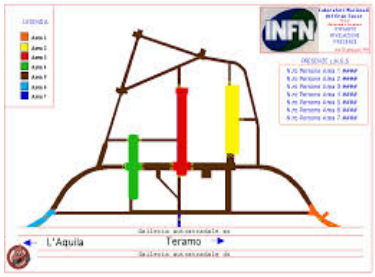
GEANT4 simulations, used for modelling the Cosmic Silence animal facilities, will complement the experimental measurements in view of a detailed evaluation of the composition and spectrum of the background radiation in the LRE and RRE

From in vitro … to in vivo models

L'Aquila University/Rome University
Reference Radiation Environment (RRE)



INFN-LNGS
Low Radiation Environment (LRE)



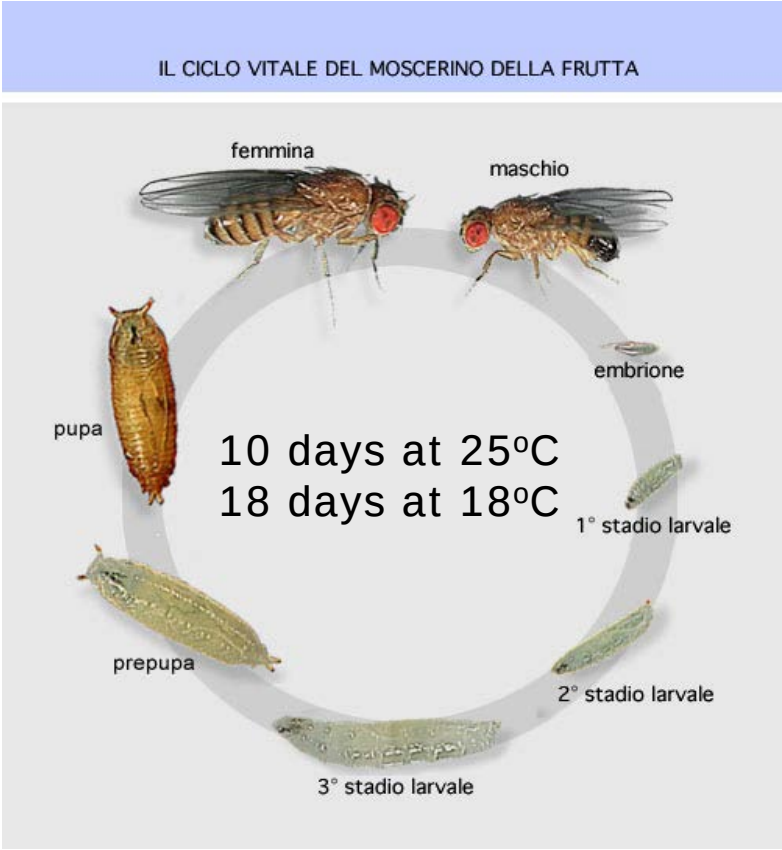
Drosophila
Melanogaster

pKZ1 mice

Animal housing and experimental procedures need to be approved by the competent Authorities (ASL, Ethical Committee, Ministero della Salute)

Drosophila as model system

- **Short life cycle (development and reproduction)**
- **High fecundity and high number of offspring**
- **Suitable for mutagenesis assays**
- **Small and easy to grow in laboratory**
- **Low overall costs**



Assays on LRE/RRE animal models

Drosophila melanogaster

pKZ1 mouse model

- **Mutation frequency at the LacZ locus**
- **Survival, fertility, locomotion activity**
- **Cell division, chromosome integrity**
- **Evaluations of expression of proteins involved in:**
  - **apoptosis**
  - **DNA breakage repair**
  - **scavenging of reactive oxygen species**

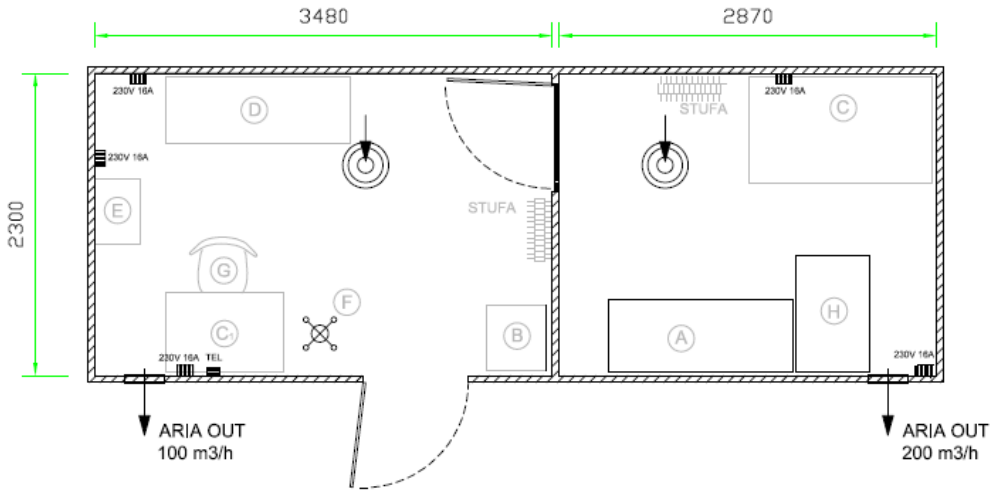
- **LacZ inversion assay**
- **Evaluations of expression of proteins involved in:**
  - **apoptosis**
  - **DNA breakage repair**
  - **scavenging of reactive oxygen species**

Radiation treatment (X-ray from linear accelerator) will be performed at the Division of Radiotherapy and Radiobiology of L'Aquila University

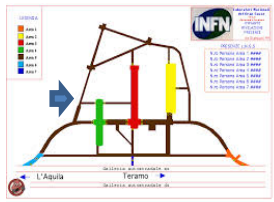
In situ tratment with chemical agents (e.g., paraquat to induce oxidative stress)

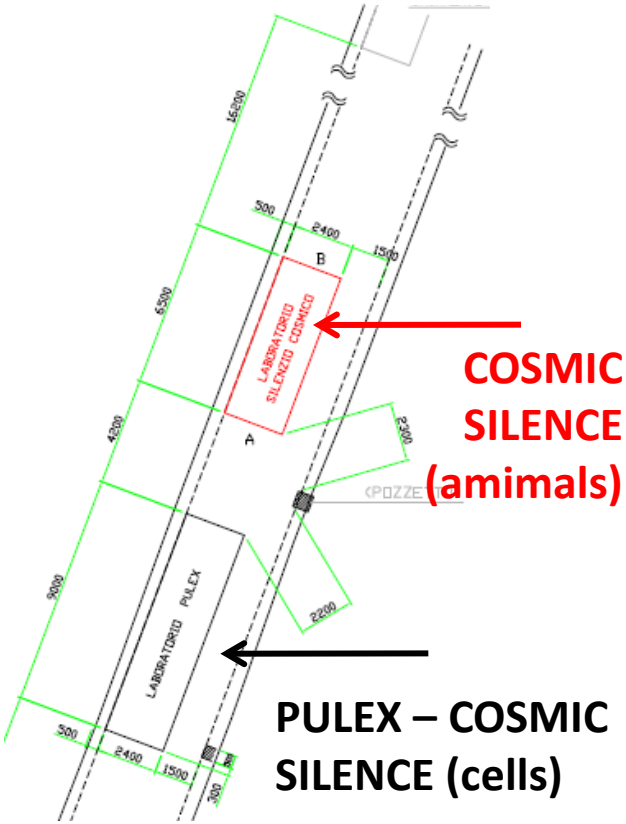
The new animal housing underground facility at LNGS

PIANTA:



LxPxH
6500x2300x2500





COSMIC SILENCE (amimals)

PULEX – COSMIC SILENCE (cells)

- **Ready-built container close to the one already installed for cell cultures (PULEX)**
  - ***Temperature and light control systems***
  - ***Ventilation system***

The facility will be realized thanks to the support given by the LNGS

It will host the equipment for organism housing, already acquired in the framework of the SILENZIO COSMICO experiment, funded by the INFN-CSN5

Our aknowledgements also go to the Centro Fermi for supporting the Pulex and Cosmic Silence experiments, in particular for the young scientist fellowships

The Cosmic Silence collaboration

ISS and INFN Roma 1 – Gr.coll. Sanità			LNGS - INFN and INFN Gr.coll. UniAq		
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Emanuela Bortolin	F	ISS	Alessandra Tessitore	B	UniAQ
Giuseppe Esposito	F	ISS	Francesca Zazzeroni	B	UniAQ
Cinzia De Angelis	F	ISS	Adriano Angelucci	B	UniAQ
Cristina Nuccetelli	F	ISS	Assunta Pompili	B	UniAQ
Mauro Belli	F		Antonella Gasbarri	B	UniAQ
Emiliano Fratini	B	Centro Fermi	Fausta Fischietti	B	Centro Fermi
Maria Balduzzi	B	ENEA	Giovanni Cenci	B	UniRM
			Marco Balata	E	LNGS
Other collaborations			Luca Ioannucci	T	LNGS
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Rebecca Omrsby	B	Flinders Univ.	**LNF - INFN**		
Pamela Sykes	B	Flinders Univ.	Adolfo Esposito	F	LNF
Francesco Cardellini	F	ENEA	Maurizio Chiti	T	LNF
Roberto Amendola	B	ENEA			
Luca Fruci	V	DVM	Luigi Satta	F	

B=biologo; F=fisico; M=medico; V=veterinario; E=ingegnere; T=tecnologo

Thank you for the attention !

Source	ISS RRE (nGy/h)	LNGS RRE (nGy/h)	LNGS LRE (nGy/h)
Directly ionizing cosmic rays (low LET)	31[a]	39[a]	negligible[c]
Neutrons from cosmic rays (high LET)	1.0[c1]	2.5[b]	negligible[c]
Total γ-rays (cosmic & terrestrial, low LET)	300[d]	34[d]	3.6[d]
^{222}Rn and daughters (high LET)	1.7[e]	0.17[e]	0.17[e]
^{40}K (internal exposure, low LET)	19[f]	19[f]	19[f]
Total (rounded)	352.7	94.7	22.8
Low-LET (rounded)	350.0	92.0	22.6
High-LET (rounded)	2.7	2.7	0.2

Previous measurements revisited

In the presence of a 5 cm Fe-shielding of the cell incubator

A pellet of 2x10^9 cells in HP-Ge spectrometer (February 2013)

after 1 month no signal above the background

(a) Evaluation based on UNSCEAR 2000 and 2008.
(b) Evaluation based on measures by Rindi et al. (1998), Bonardi et al. (2010) and Olsher et al. (2010) applying the Kerma factors for water listed in ICRU Report 46 (1992).
(c) As above, applying the experimental reduction factors of the rock coverage; (c1) value from (b) taking into account altitude difference between LNGS and Rome
(d) TLD measurements
(e) Calculation based on the application of the model by Jostes et al. (1991) to the measured Rn concentration
(f) Evaluated by equating the 40K concentration in cells to that of the human body and applying the data from UNSCEAR 2000

Expression of genes involved in the protection from oxidative damage

Summary

Catalase (Cat): catalyzes the decomposition of hydrogen peroxide to water and oxygen

Superoxide dismutases (Sod): class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide

Glutathione peroxidase 1 (**Gpx1**): works in the detoxification of hydrogen peroxide and is one of the most important antioxidant enzymes in humans

Extracellular glutathione peroxidase (**Gpx3**): works in hydrogen peroxide detoxification in the extra-cellular compartment

Phospholipid hydroperoxidase (Gpx4):

uses lipid-hydroperoxide as substrate; protects cells against membrane lipid peroxidation

Selenium binding protein (Sbp1):
down-regulates GPx_s activity removing selenium

After 1 month of continuous culture

	Reference Lab (ISS)	Underground Lab (LNGS)
Cat	-	↓
Sod	↑	↓
Gpx1	↑	-
Gpx2	-	-
Gpx3	-	↓
Gpx4	↑	-
Sbp1	↓	↑