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Genetic Manipulation, Gene Silencing and Biomarker Development in Multiple Experimental Models.

Presentata da: **Marco De Cecco**

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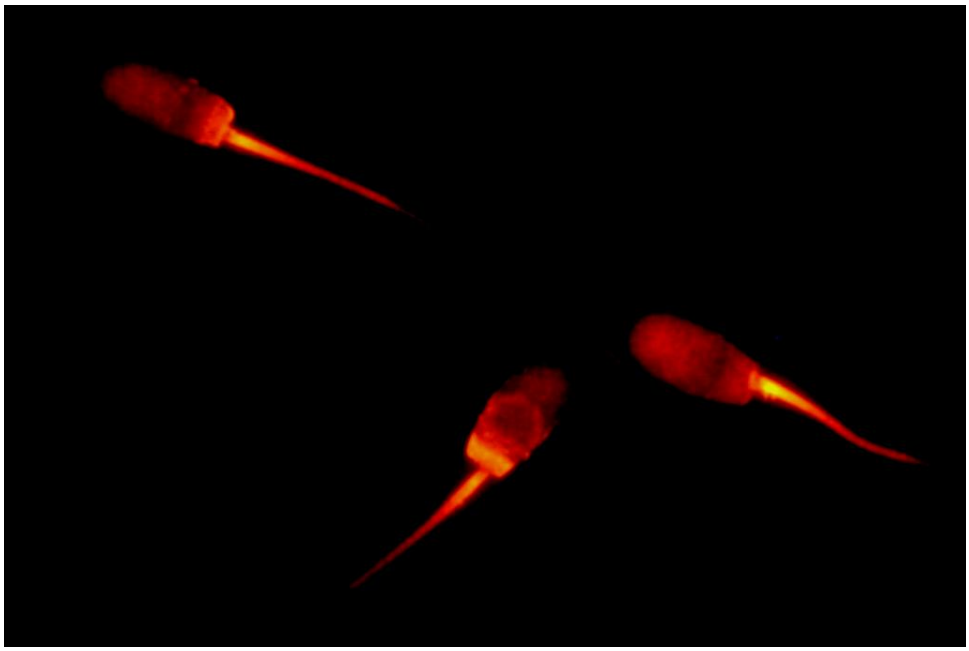

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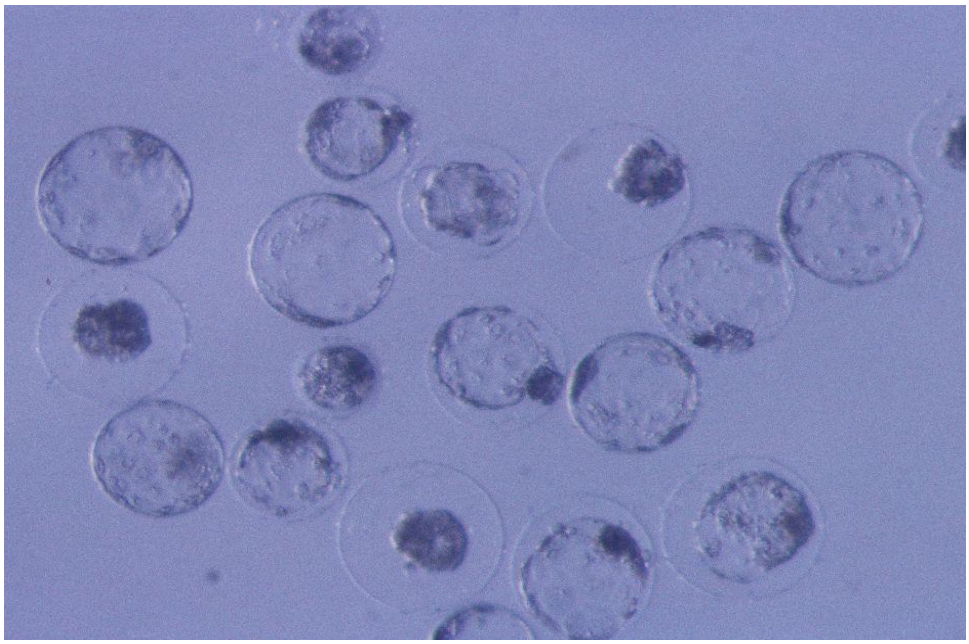

Part 1 – Generating transgenic animals via Sperm Mediated Gene Transfer

Introduction: approaching the single step transgenesis

Transgenesis

A transgenic animal can be defined as one having any specific, targeted genetic modification (Dunn et al., 2005).

Virtually every DNA sequence maybe expressed in transgenic animals, and the recombinant DNA techniques allow binding genic sequence to regulatory elements in order to consent exogenous gene expression in a way tissue-specific or even ubiquitary. The exogenous DNA is then inherited by the offspring through the germ line. Literature data set the creation of the first transgenic mouse in 1980, by Gordon J.W. et al.; lately many other groups reached this goal, proving the efficiency of the transgenesis and the conservation in the following generations (Brinster et al., 1981; Constantini & Lacy, 1981; Wagner et al., 1981).

The production of transgenic animals responds to different aims, in the Xenome project – on which I spent most of the work - the purpose is the engineering of the porcine genome for xenotransplantation studies in primates (Xenome integrated project, 2006).

Sperm Mediated Gene Transfer

In the past fifteen years the laboratory of professors Bacci ML and Forni M focused on a transgenesis technique: the SMGT. The main idea underlying this technique is the intrinsic ability of sperm cells to bind and internalise exogenous DNA and to transfer it into the oocyte at fertilisation (Bacci, 2007).

The founding that sperms were able to bind to specific genic sequence and carry them into the oocyte is dated back to 1971 (Brackett et al., 1971). But

then there were no further works in that direction for about eighteen years, when the preliminary data found confirmation in sea-urchin (Arezzo, 1989) and in mouse (Lavitrano et al., 1989).

Subsequently, SMGT was successfully adapted and optimised for use in large animals by a tight collaboration with DiMorFiPa research group (Sperandio et al., 1996). From then onwards more success was obtained: human decay accelerating factor (hDAF) transgenic pig lines had been produced with high efficiency (Lavitrano et al., 2002; 2003): those transgenic pigs showed good protection against a hyperacute rejection challenge in ex-vivo experiments (Smolenski et al., 2007).

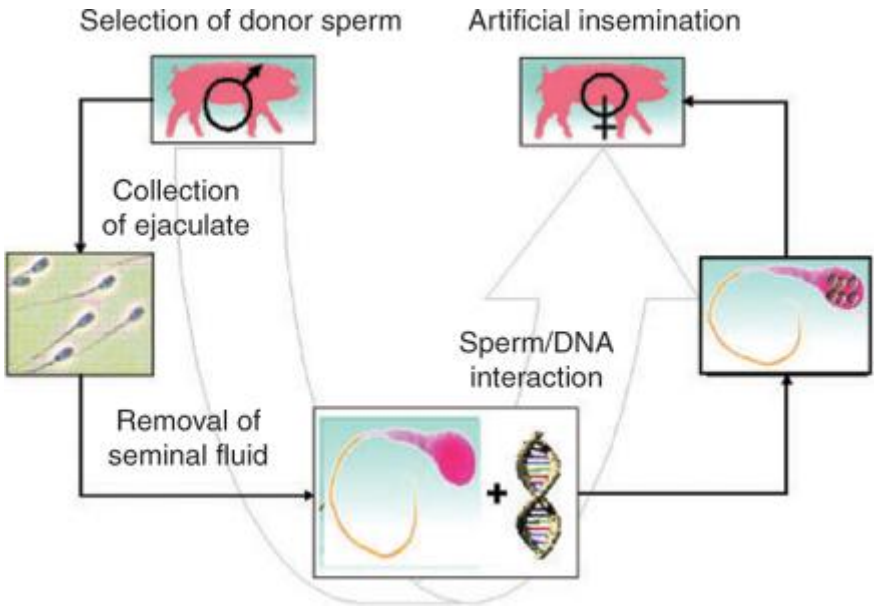

Figure 1: Sperm-mediated gene transfer in the pig. (Lavitrano et al., 2007)

It has also been demonstrated that SMGT is also suitable for the transformation of the animal genome with a non viral episomal vector (Manzini et al., 2006). Other researchers have also proven the broad applicability of this technique to different animal species, from sea urchin to cattle (Wall, 2002), so SMGT can be considered as a powerful and potentially universal technique (Bacci, 2007).

The exogenous DNA molecules bind to the sperm cell's head in the subacrosomal region and in the proximity of the equatorial area. Once bound to the cell membrane, DNA molecules are taken up (Francolini et al. 1993). The process of DNA binding and internalisation is not a random event. The exogenous DNA interacts with DNA-binding proteins (DBPs) of 30–35 kDa, which are present on the sperm cell surface (Lavitrano et al. 1992; Zani et al. 1995) and it has been demonstrated that major histocompatibility complex (MHC) class II and CD4 molecules also play a role in the process of sperm/DNA interaction (Lavitrano et al. 1997b). In fact, sperm cells from MHC class II-knockout mice have a reduced ability to bind DNA compared with sperm cells from wild-type animals (Mori et al. 1990; Wu et al. 1990). Conversely, spermatozoa from CD4-knockout mice are fully capable of binding exogenous DNA, yet lose the ability to internalise it. To further support the role of CD4 in DNA transfer, it has been shown that nuclear internalisation of exogenous DNA is prevented in wild-type sperm cells pre-incubated with anti-CD4 monoclonal antibodies (mAbs). Thus, although not present in mature sperm cells, MHC class II expression appears to be required during spermatogenesis to produce sperm cells capable of taking up foreign DNA, whereas CD4 molecules present on sperm cells mediate the nuclear internalisation of sperm-bound DNA (Lavitrano et al. 1997b).

The SMGT molecular model hasn't been in the spotlight for while, and then recently Wang et al. (2011) described a correspondence between CD4 polymorphisms and ability of sperm to internalize exogenous DNA. This reinforces the previous finding and anticipates CD4 as target for future and definitive studies on the SMGT model.

Nevertheless mature spermatozoa are naturally protected against the intrusion of foreign DNA molecules; in fact, it has been identified a factor (inhibitory factor 1, IF-1) present in the seminal fluid of mammals that blocks the binding of exogenous DNA to sperm cells. This factor also exerted a powerful inhibitory effect on DNA uptake in sperm cells of heterologous species. The DNA Binding Proteins (DBPs) appeared to be the specific target

through which the inhibition is mediated because, in the presence of the inhibitory factor, the DBPs lose their ability to bind exogenous DNA (Zani et al. 1995). Thus, the interaction of exogenous DNA with sperm cells does not appear to be a casual event but, in contrast, relies on a molecular mechanism based on the cooperation of specific protein factors. In the absence of IF-1, DBPs are able to interact with DNA and the complex can translocate the DNA into the cell in a CD4-dependent manner (Lavitrano et al. 1997b). Thus, it is of utmost importance that seminal fluid is removed from sperm samples by extensive washing as soon as possible after ejaculation.

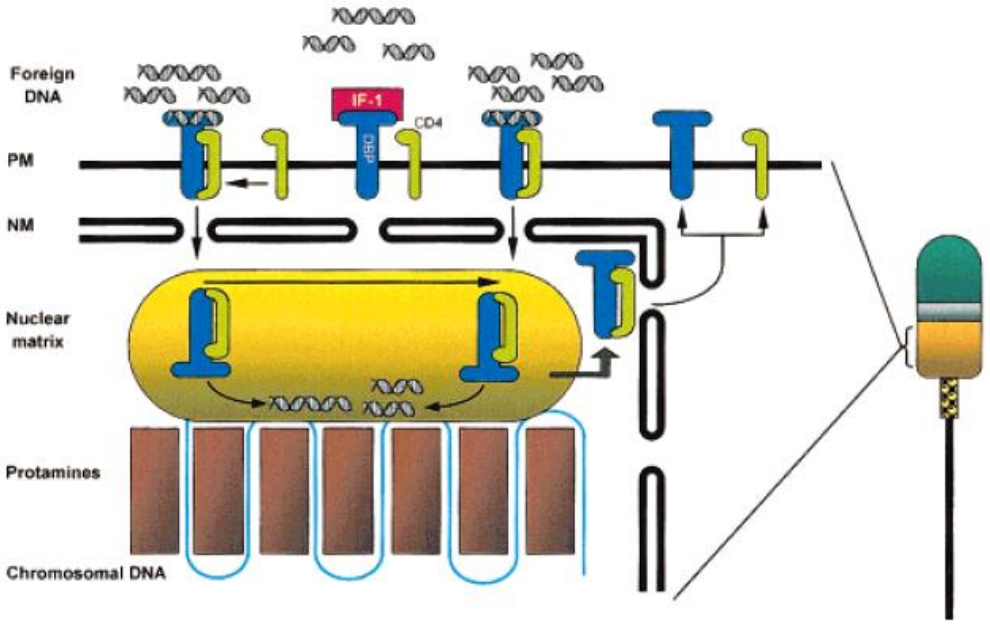

Figure 2: Schematic representation of the process of internalization of foreign DNA in the sperm nucleus (Spadafora et al., 1998).

The attention on the seminal plasma complete removal has always been a crucial part of SMGT experiments as shown in the recent publications by Zhao et al. on goat spermatozoa (Zhao et al., 2011), Collares et al. in chickens (Collares et al., 2011), and particularly in catfish (Rhamdia quelen) spermatozoa, where Campos et al. observed a strong DNase activity in seminal plasma (Campos et al., 2011).

Even though much effort has been spent on studying the relationship between SMGT and seminal plasma, the Interfering Factor I is yet to be uniquely identified.

After DNA–sperm cell interaction and internalisation, the subsequent step involves integration of the exogenous DNA into the genome.

It is not known whether the DNA integration event happens before fertilisation. Interestingly, using the SMGT technique, it has been found that foreign DNA sequences are tightly bound to the sperm nuclear scaffold and that integration of the exogenous DNA occurs preferentially in the long interspersed nuclear element type-1 elements, repeated sequences interspersed into the genome. Moreover the presence of a topoisomerase II consensus sequence at one end of the integration site suggests a possible role for this enzyme in the integration process (Spadafora et al., 1998).

These considerations characterize the SMGT as a very interesting technique that still requires deep molecular characterization but at the same time, guarantees results in the production of transgenic animals. In a consistent part of my work we focused on the developing of new methodologies for SMGT, particularly in swine, and their application in the *in vivo* and *in vitro* production of transgenic animals within the Xenome Project.

Reproductive biotechnologies

Several systems have been established to generate embryos in vitro, however the efficiency is still low. The major problems include improper oocyte maturation, both nucleus and cytoplasmic, and polyspermy. The importance of the development and identification of defined in vitro conditions for oocyte maturation and fertilization should not be underestimate, since most reproductive technologies rely on these basic techniques (Sun & Nagai, 2003).

In Vitro Maturation

Maturation of the oocytes includes two aspects: nuclear maturation and cytoplasmic maturation. Generally an oocyte is regarded to be matured when the first polar body is extruded (nuclear maturation) and the oocyte is arrested at the MII stage.

Although nuclear matured oocytes can be fertilized, they may be developmentally incompetent due to the deficiency in some cytoplasmic maturation. Thus, the developmental potential requires synchronous nuclear maturation and cytoplasmic maturation (Sun & Nagai, 2003).

In Vitro Maturation (IVM) protocols have the aim to recreate artificially the most favorable conditions to allow cytoplasmatic and nuclear maturation of the oocyte.

In swines, IVM applied to female gametes is based on two phases, for a total duration of about 44-48 hours.

The first phase is 24 hours long, and is characterized by the presence of dibutyrril-cAMP, eCG and hCG: the whole medium is called IVM maturation medium type B.

The db-cAMP is a synthesis analogue of cAMP added to culture medium; it is a tiny molecule able to penetrate cell and nuclear membranes, when it reaches the nucleus keeps the meiotic process blocked untill it is removed by nuclear exposition to hCG gonadotropin, that recreates LH pre-ovulatory peak.

hCG and eCG are both corionic gonadotropin, respectively derivating from “human” and “equine”. Their action is very important, because they act like gonadotropy hormones FSH (Follicule Stimulating Hormone) and LH (Luteinizing Hormone) inside an organism.

eCG is FSH-like: its target are the follicular somatic cells of Teca and Granulosa, and promotes the development of cumulus structures deputated to protect and feed the egg cell. During the first phase of IVM process, this hormone maintains working the gap-junction between somatic cells and oocyte, promoting the circulation of nutritive substances to oocyte.

hCG is LH-like: its presence allows the meiosys restart when db-cAMP levels decrease.

These molecules allow the cell to adapt to the new environment and to prepare to next steps in maturation.

The second maturation phase is 24 hours long, and takes place in the same medium, IVM maturation medium, but without db-cAMP and hormones neither. This is the so called IVM maturation medium type A.

Meiotic maturation is fully reached at the end of this phase, gap-junction broke up and somatic cells start jaluronic acid production, in order to expand more and more the cumulus. These transformation are index of the maturation.

At the end of the two phases swine oocyte should have concluded both maturation, cytoplasmatic and nuclear.

In Vitro Fertilization

In Vitro Fertilization (IVF) allows the fertilization event in a in vitro system. Fertilization requires mature oocytes: both cytoplasm and nucleus (at MII stadium). To evaluate cytoplasmatic maturation before fertilization is quite complex: it is possible only in a second time, based on effects subsequent to fertilization event. One of the most important parameters is the ability of a mature oocyte to prevent polyspermy and to protect normospermy. It is such a critical step because polyspermy in swine is quite common.

Besides, oocytes must be able to de-condense sperm head, in order to create male pronucleus, essential for singamy.

Sperm Sorting

It is estimated that as many as 60,000 offspring, mostly cattle, have been produced in the past 10 years using AI or some other means of transport with spermatozoa sexed by flow cytometric sperm sorting and DNA as the marker of differentiation.

Numerous works demonstrated that the only reliable marker for the efficient separation of X- and Y-chromosome bearing spermatozoa is DNA (Johnson et al, 2005). This method is now used worldwide and was known from the beginning as the Beltsville Sperm Sexing Technology. A fluorescent dye (Hoechst 33342) is used to stain DNA and the method is based on the separation of sperm using flow cytometric sorting to sort fluorescently labeled sperm based on their relative content of DNA within each population of X- and Y-spermatozoa. The technology is being applied in livestock, laboratory animals, and zoo animals, even in humans, with a success rate of 90-95% in shifting the sex ratio of offspring. Delivery of sexed sperm to the site of fertilization varies with species. Although sperm of all species can be sorted with high purity, achieving pregnancies with the low numbers of sperm needed for commercial application remains particularly elusive in swine.

With these premises it is of crucial interest to couple a transgenesis method as SMGT with sperm sorting: the potential applications in the field of animal productions are large. With an offspring of defined sex it would be much easier to undercut the costs of production of transgenic, maximizing the number of transgene-carrying animals and at the same time being able to mate all the subjects.

Appendix 1 - Spermatozoa and DNA

Assessing quality parameters in semen

The first part of the work focused on the assessment of optimal quality parameters during the SMGT treatment: the aim of the study was to investigate the influence of SMGT treatment and exogenous DNA uptake on sperm quality.

In our publication we demonstrated that SMGT-treated spermatozoa retain good quality and fertilization for at least twenty four hours.

Article:

Sperm-mediated gene transfer-treated spermatozoa maintain good quality parameters and in vitro fertilization ability in swine.

Bacci ML, Zannoni A, **De Cecco M**, Fantinati P, Bernardini C, Galeati G, Spinaci M, Giovannoni R, Lavitrano M, Seren E, Forni M.

Theriogenology. 2009 Dec;72(9):1163-70. Epub 2009 Sep 19.

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Sperm-mediated gene transfer–treated spermatozoa maintain good quality parameters and in vitro fertilization ability in swine

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Abstract

A simple and efficient method for producing multitransgenic animals is required for medical and veterinary applications. Sperm-mediated gene transfer (SMGT) is an effective method for introducing multiple genes into pigs (Sus, Sus scrofa). The major benefits of this technique are the high efficiency, low cost, and ease of use compared with that of other methods: Sperm-mediated gene transfer does not require embryo handling or expensive equipment. The aim of this study was to investigate the influence of SMGT treatment and exogenous DNA uptake on sperm quality. Even after a coincubation with a 20-fold larger amount (100 μg/mL) of DNA than usual (5 μg/mL), sperm quality parameters were not significantly affected, confirming the hypothesis that the SMGT protocol itself or the amount of bound DNA do not compromise the possibility of an extended employment of SMGT. More importantly, we found that semen used for in vitro fertilization 24 h after DNA uptake gave good cleavage (60% vs. 58%, treated vs. control) and developmental rates definitely positive (41% vs. 48%, treated vs. control). These good results are connected to a competitive efficiency of transformation (62%) due to the numerous improvements in SMGT technique. We demonstrate that SMGT-treated spermatozoa retain good quality and fertilization potential for at least 24 h, expanding the possibility to apply transgenesis in field conditions in swine, where the greatest hurdles are fertilization timing and plain procedure.

Keywords: Fertility; Semen storage; Sperm-mediated gene transfer; Swine; Transgenesis

1. Introduction

Sperm-mediated gene transfer (SMGT) for creation of transgenic animals is an emerging technique as documented by the increasing number of scientific publications on this topic in many species such as mammals, birds [1], amphibians [2], and some invertebrates [3] and insects [4]. In past decades, we optimized the technique in large animal species (i.e., bovine and swine [5]), then we obtained many successful productions of transgenic pigs for a human inhibitor of complement cascade (hDAF) [6,7], and subsequently we expanded the SMGT technique optimizing a one-step multitransgene pig production [8] with successful use of a nonviral episomal vector for the pig genome modification [9]. The conjoint application of laparoscopic insemination at the utero-tubal junction [10] allowed also a 10-fold reduction of spermatozoa in the insemination doses in comparison with those used in artificial insemination (AI) in swine.

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Development of a method for screening DNA uptake in sperm

In order to consolidate the use of SMGT in transgenesis with multiple exogenous constructs, we developed a method that allows a real time screening of DNA uptake. This work can be considered as a breakthrough since the previous standard approach was a radiolabeled ^{3}H-DNA coincubation, that prevent the screening of more than one construct.

Our work, published on the Journal of Biomolecular Techniques, relies on fluorescent-specific peaks for each construct, and their diminution due to internalization during the sperm – DNA coincubation phase. This is particularly interesting for experiments, like our Xenome project, where more than one type of plasmid is involved and they are all required for a successful transgenesis.

Article:

> ***A method for uptake quantification of multiple fluorescent DNAs in boar semen as an alternative to radiolabeling.***

De Cecco M, Zannoni A, Bernardini C, Lavitrano M, Bacci ML, Forni M.

J Biomol Tech. 2010 Jul;21(2):61-5.

PMID: 20592868

URL: http://www.ncbi.nlm.nih.gov/pubmed/20592868

COMMUNICATION

A Method for Uptake Quantification of Multiple Fluorescent DNAs in Boar Semen As an Alternative to Radiolabeling

Marco De Cecco,[1,] Augusta Zannoni,[1] Chiara Bernardini,[1] Marialuisa Lavitrano,[2] Maria Laura Bacci,[1] and Monica Forni[1]*

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Sperm-mediated gene transfer (SMGT) is a simple and efficient method for producing multitransgenic organisms. Until now, the exogenous DNA uptake efficiencies have been quantified, performing coincubation of spermatozoa with ^{3}H-DNA. This method has significant limitations; from a researcher's point of view, radioactivity-based experiments are hazardous and require specialistic skills, and in technical analysis, the signal does not allow the simultaneous discrimination of two or more types of labeled constructs. Considering these remarkable points, the present work aims to develop a method for differential uptake quantification of various transgenes alternative to the use radioactive material. The main approach relies on fluorescent-specific peaks for each construct, and their diminution during the sperm–DNA-coincubation phase. The obtained results were confirmed by real-time PCR analysis and fluorescence microscopy imaging. This method becomes of primary importance when the SMGT technique has to be applied on various constructs, as it allows preliminary conclusions to be drawn about multiple transgenesis events and to approach further research about eventual sperm membrane preferences in sequences or structures for constructs.

Key Words: gene transfer technique, fluorescence, uptake, spermatozoa

INTRODUCTION

Sperm-mediated gene transfer (SMGT) for creation of transgenic animals is an emerging technique, as documented by the increasing number of scientific publications about this topic in many species.[1,2] In past decades, we optimized the technique in large animal species, i.e., bovine and swine,[3] and then we obtained many successful productions of transgenic pigs for the human inhibitor of complement cascade (human decay accelerating factor).[4,5]

Until now, the exogenous DNA uptake efficiencies have been quantified, performing coincubation of spermatozoa with ^{3}H-DNA and evaluating the resulting cpm in a β-counter before and during coincubation. The literature shows how the method of uptake quantification using DNA labeled with tritium proved to be absolutely reliable and effective.[6,7] This, however, has a limitation inherent to the technology itself; the signal does not allow the simultaneous discrimination of two or more types of labeled constructs. Moreover, radioactivity is well known to be biologically hazardous, so it is desirable to replace it whenever possible.

Given the feasibility demonstrated in our previous studies, with the creation of multitransgenic pigs "one-step" through SMGT,[8] the need to ascertain the distribution of the different constructs in the sperm population becomes of prime importance. Such a method should facilitate the monitoring of the uptake of multiple constructs during SMGT spermatozoa—exogenous DNA-coincubation phase—and at the same time, it should not cause significant side-effects on sperm. Experimental procedures should maintain spermatozoa close to SMGT operating conditions to gain reliable data. Considering these requirements, the present work aims to develop and validate a method for fluorescent uptake quantification of various transgenes by pig spermatozoa in real time.

MATERIALS AND METHODS

Fluorescent DNA Labeling

Plasmids pT7T3-18U (GE Healthcare, Pittsburgh, PA, USA; 2.8 kb long), pGEM-9Zf(−) (Promega, Madison, WI, USA; 2.9 kb long), and pUC18 (Fermentas, Vilnius, Lithuania; 2.7 kb long) were used in sperm/circular DNA uptake analysis.

Fluorescent labels, specific for each construct, were

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Appendix 2 - Production and genotyping of porcine blastocysts

Use of SMGT to generate multiple transgenic embryos

The expertise gained in the previous work was exploited in the production of 1-step genetically modified embryos. The three constructs carried genes involved in the modulation of inflammatory processes (hHO1, hCD39, and hCD73).

The results, published on Transplant Proc, suggest that this system allowed an effective preliminary test of transgenesis, majorly reducing the number of animals used in the experiments and fulfilling important ethical issues.

Article:

> ***In Vitro Production of Multigene Transgenic Blastocysts via Sperm-Mediated Gene Transfer Allows Rapid Screening of Constructs to Be Used in Xenotransplantation Experiments.***

Vargiolu A, Manzini S, **De Cecco M**, Bacci ML, Forni M, Galeati G, Cerrito MG, Busnelli M, Lavitrano M, Giovannoni R.

Transplant Proc. 2010 Jul-Aug;42(6):2142-5.

PMID: 20692428

URL: http://www.ncbi.nlm.nih.gov/pubmed/20692428

ELSEVIER

In Vitro Production of Multigene Transgenic Blastocysts via Sperm-Mediated Gene Transfer Allows Rapid Screening of Constructs to Be Used in Xenotransplantation Experiments

A. Vargiolu, S. Manzini, M. de Cecco, M.L. Bacci, M. Forni, G. Galeati, M.G. Cerrito, M. Busnelli, M. Lavitrano, and R. Giovannoni

ABSTRACT

Multigene transgenic pigs would be of benefit for large animal models and in particular for xenotransplantation, where extensive genetic manipulation of donor pigs is required to make them suitable for organ grafting to humans. We have previously produced multitransgenic pigs via sperm-mediated gene transfer (SMGT) using integrative constructs expressing 3 different reporter genes. The aim of the present work was to evaluate the efficacy and safety of using 3 integrative constructs carrying 3 different human genes involved in the modulation of inflammatory responses. We developed an in vitro fertilization system to demonstrate that SMGT can be used to efficiently produce multigene transgenic embryos through a 1-step genetic modification using multiple integrative constructs each carrying a different human gene involved in the modulation of inflammatory processes (hHO1, hCD39, and hCD73). The results suggest that this system allowed an effective preliminary test of transgenesis optimization, greatly reducing the number of animals used in the experiments and fulfilling important ethical issues. We performed 5 in vitro fertilization experiments using sperm cells preincubated with all 3 integrative constructs. A total of 1,498 oocytes were fertilized to obtain 775 embryos, among which 340 further developed into blastocysts. We did not observe any toxicity related to the transgenesis procedure that affected normal embryo development. We observed 68.5% transgenesis efficiency. Blastocysts were 48% single, 31% double, and 21% triple transgenic.

To date, sperm-mediated gene transfer (SMGT) experiments have successfully produced transgenic offspring with high efficiency when performed with linear plasmid DNA that integrates into the sperm genome. We have previously taken advantage of SMGT to efficiently produce transgenic pigs that express human decay accelerating factor, which has been shown to help overcome the first rejection barrier in pig-to-primate transplantation models.[1] Moreover, we have also produced a multigene transgenic pig model that expresses 3 reporter genes.[2] Because we were interested in studying the molecular mechanisms of acute rejection in xenotransplantation, we produced 3 constructs, each containing a human gene that is considered to be a key modulator of inflammation: heme oxygenase 1 (hHO1), ectonucleoside triphosphate diphosphohydrolase 1 (hENTPD1, also known as hCD39), and 5′-ecto-nucleotidase (hNT5E, also known as hCD73). The aim of this study was to produce multigene transgenic blastocysts via in vitro fertilization (IVF) combined with SMGT to obtain rapid screening of constructs for transgenesis experiments to produce genetically modified pigs for

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Appendix 3 - Production of transgenic blastocysts of predetermined sex

Use of sperm sorting techniques and SMGT to generate transgenic embryos

The final part of the work on sperm mediated gene transfer focused on proving the possibility to positively couple sperm sexing with SMGT, in order to speed up the assessment of homozygous lines of transgenic pigs.

After much work in the tuning of the protocol, the method we developed and published on Theriogenology allows to produce transgenic swine blastocysts of pre-determined gender.

Article:

Coupling sperm mediated gene transfer and sperm sorting techniques: a new perspective for swine transgenesis.

De Cecco M, Spinaci M, Zannoni A, Bernardini C, Seren E, Forni M, Bacci ML.

Theriogenology. 2010 Sep 15;74(5):856-62. Epub 2010 May 26.

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Coupling sperm mediated gene transfer and sperm sorting techniques: a new perspective for swine transgenesis

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Abstract

Flow cytometric separation of X and Y chromosome-bearing spermatozoa has been demonstrated to be effective in pigs, allowing the use of boar sexed semen in *in vitro* trials. Sperm Mediated Gene Transfer (SMGT) is a widely used and efficient technique for the creation of transgenic animals. The present research intended to prove that it is possible to associate sperm sexing with the SMGT technique in order to speed up the assessment of homozygous lines of transgenic pigs. In the first experiment, the sorting protocol was modified in order to obtain the highest DNA uptake by sorted spermatozoa. In the second experiment, spermatozoa that had undergone only sperm sorting, only SMGT, or both procedures (Sorted-SMGT) were used for in *in vitro* fertilization of *in vitro* matured oocytes. In the third experiment, transformed blastocysts of the desired gender (male) were obtained with Sorted-SMGT in an *in vitro* fertilization trial. The method we developed here allowed us to produce transgenic swine blastocysts of pre-determined gender, giving a positive answer at the aim to couple SMGT and sperm sorting in swine, obtaining fertile spermatozoa able to produce transgenic embryos of pre-determined gender.

Keywords: Swine; Sperm Mediated Gene Transfer; Sperm sexing; *In vitro* fertilization; Transgenesis

1. Introduction

Sperm-mediated gene transfer (SMGT) for the creation of transgenic animals of many species is a widely used technique, as documented by a number of scientific publications [1,2]. The technique has been optimized in large animal species, i.e., bovine and swine [3], and we obtained many successful productions of transgenic pigs for a human inhibitor of complement cascade (hDAF) [4]. Subsequently, we expanded the SMGT technique optimizing a one-step multitransgene pig production [5] and with the successful use of a non viral episomal vector for the genome modification [6]. The application of transgene techniques in swine species is strongly sustained by renewed interest in pigs as organ donors for xenotransplantation [7,8], in the expanded use of genetic modified pigs as valuable models for biomedical studies [9], and in the use of transgenic swine as bioreactors [10].

Due to the long life cycle and physical dimensions of the pig, the cross programs necessary to obtain homozygous transgenic pig lines are really a treaty aspect in developing useful transgenic pig models. A consistent shortcut could be represented by technology that allows us to obtain high numbers of transgenic males in the first generation. The technology could then

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Bibliography

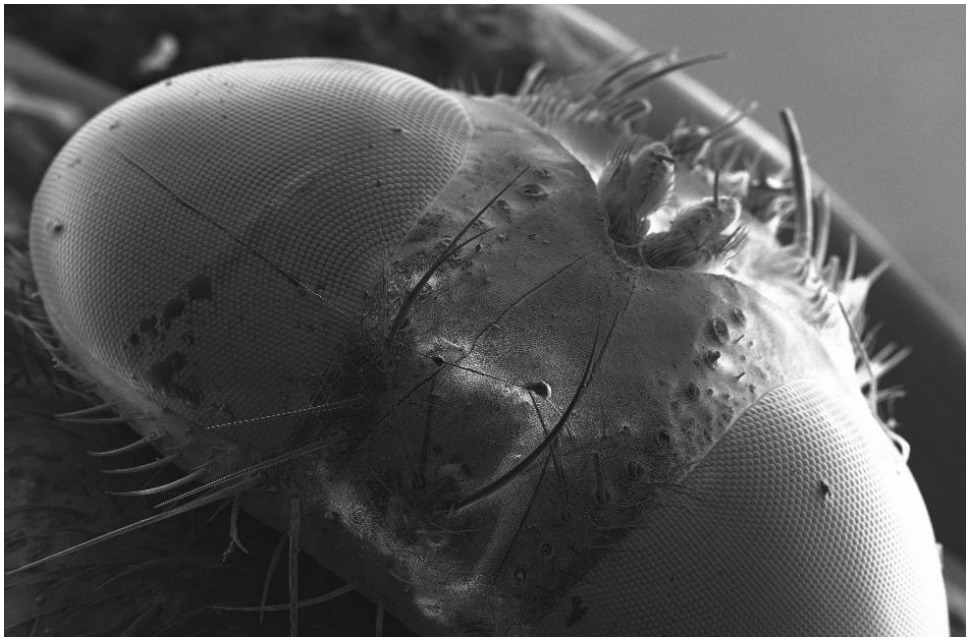
1. Arezzo F. Sea urchin sperm as a vector of foreign genetic information. Cell Biology International Reports 1989 Apr;13(4)391-404.
2. Bacci ML, Zannoni A, De Cecco M, Fantinati P, Bernardini C, Galeati G, Spinaci M, Giovannoni R, Lavitrano M, Seren E, Forni M. Sperm-mediated gene transfer-treated spermatozoa maintain good quality parameters and in vitro fertilization ability in swine. Theriogenology. 2009 Dec;72(9):1163-70. Epub 2009 Sep 19.
3. Bacci ML. A brief overview of transgenic farm animals. Vet Res Commun. 2007 Aug;31 Suppl 1:9-14.
4. Brackett BG, Baranska W, Sawicki W, Koprowski H. Uptake of heterologous genome by mammalian spermatozoa and its transfer to ova through fertilization. Proc Natl Acad Sci U S A. 1971 Feb;68(2):353-7.
5. Brinster RL, Chen HY, Trumbauer M, Senear AW, Warren R, Palmiter RD. Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. Cell. 1981 Nov;27(1 Pt 2):223-31.
6. Campos VF, Amaral MG, Seixas FK, Pouey JL, Selau LP, Dellagostin OA, Deschamps JC, Collares T. Exogenous DNA uptake by South American catfish (Rhamdia quelen) spermatozoa after seminal plasma removal. Anim Reprod Sci. 2011 Jun;126(1-2):136-41. Epub 2011 May 24.c
7. Collares T, Campos VF, De Leon PM, Cavalcanti PV, Amaral MG, Dellagostin OA, Deschamps JC, Seixas FK. Transgene transmission in chickens by sperm-mediated gene transfer after seminal plasma removal and exogenous DNA treated with dimethylsulfoxide or N,N-dimethylacetamide. J Biosci. 2011 Sep;36(4):613-20.

8. Costantini F, Lacy E. Introduction of a rabbit beta-globin gene into the mouse germ line. Nature. 1981 Nov 5;294(5836):92-4.
9. De Cecco M, Spinaci M, Zannoni A, Bernardini C, Seren E, Forni M, Bacci ML. Coupling sperm mediated gene transfer and sperm sorting techniques: a new perspective for swine transgenesis. Theriogenology. 2010 Sep 15;74(5):856-62. Epub 2010 May 26.
10. De Cecco M, Zannoni A, Bernardini C, Lavitrano M, Bacci ML, Forni M. A method for uptake quantification of multiple fluorescent DNAs in boar semen as an alternative to radiolabeling. J Biomol Tech. 2010 Jul;21(2):61-5.
11. Dunn DA, Pinkert CA, Kooyman DL. Foundation Review: Transgenic animals and their impact on the drug discovery industry. Drug Discov Today. 2005 Jun 1;10(11):757-67.
12. Francolini M, Lavitrano M, Lamia CL, French D, Frati L, Cotelli F, Spadafora C. Evidence for nuclear internalization of exogenous DNA into mammalian sperm cells. Mol Reprod Dev 1993 34, 133–139.
13. Johnson LA, Rath D, Vazquez JM, Maxwell WM, Dobrinsky JR. Preselection of sex of offspring in swine for production: current status of the process and its application. Theriogenology. 2005 Jan 15;63(2):615-24.
14. Lavitrano M, Bacci ML, Forni M, Lazzereschi D, Di Stefano C, Fioretti D, Giancotti P, Marfè G, Pucci L, Renzi L, Wang HJ, Stoppacciaro A, Stassi G, Sargiacomo M, Sinibaldi P, Turchi V, Giovannoni R, Della Casa G, Seren E and Rossi G. Production by sperm-mediated gene transfer of human decay accelerating factor (hDAF) transgenic pigs for xenotransplantation. Proceedings of National Academy of Science of the United States of America. 2002 99(22), 14230–14235.
15. Lavitrano M, Camaioni A, Fazio VM, Dolci S, Farace MG, Spadafora C. Sperm cells as vectors for introducing foreign

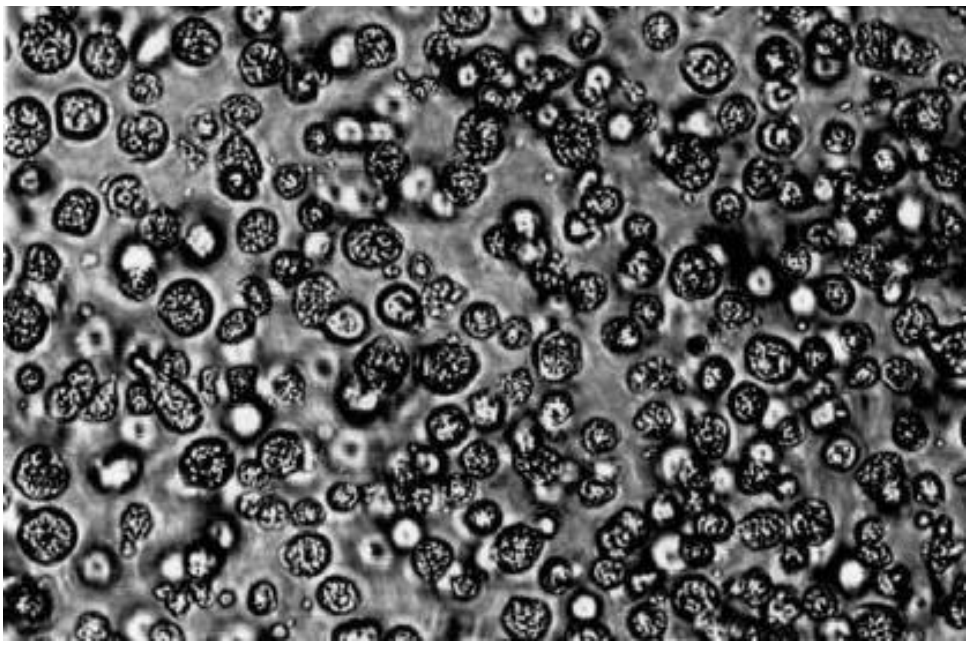
DNA into eggs: genetic transformation of mice. Cell. 1989 Jun 2;57(5):717-23.

16. Lavitrano M, Forni M, Bacci ML, Di Stefano C, Varzi V, Wang H, Seren E. Sperm mediated gene transfer in pig: Selection of donor boars and optimization of DNA uptake. 2003 Mol Reprod Dev 64:284–291.
17. Lavitrano M, French D, Zani M, Frati L, Spadafora C. The interaction between exogenous DNA and sperm cells. Mol. Reprod. Dev. 1992 31, 161–169.
18. Lavitrano M, Maione B, Forte E, Francolini M, Sperandio S, Testi R, Spadafora C. The interaction of sperm cells with exogenous DNA: a role of CD4 and major histocompatibility complex class II molecules. Exp. Cell Res. 1997b 233, 56–62.
19. Manzini S, Vargiolu A, Stehle IM, Bacci ML, Cerrito MG, Giovannoni R, Zannoni A, Forni M, Donini P, Papa M, Lipps HJ, Lavitrano M. Genetically modified pigs produced with a non viral episomal vector. Proceedings of National Academy of Science of the United States of America. 2006 103(47), 17672–17677.
20. Smolenski RT, Forni M, Maccherini M, Bacci ML, Slominska EM, Wang H, Fornasari P, Giovannoni R, Simeone F, Zannoni A, Frati G, Suzuki K, Yacoub MH, Lavitrano M. Reduction of hyperacute rejection and protection of metabolism and function in hearts of human decay accelerating factor (hDAF)-expressing pigs. Cardiovasc Res. 2007 Jan 1;73(1):143-52. Epub 2006 Nov 6.
21. Spadafora C. (1998). Sperm cells and foreign DNA: a controversial relation. BioEssays 20, 955–964.
22. Sperandio S, Lulli V, Bacci ML, Forni M, Maione B, Spadafora C and Lavitrano M. Sperm mediated DNA transfer in bovine and swine species. Animal Biotechnology. 1996 7, 59–77.

23. Sun QY, Nagai T. Molecular mechanisms underlying pig oocyte maturation and fertilization. J Reprod Dev. 2003 Oct;49(5):347-59.
24. Vargiolu A, Manzini S, de Cecco M, Bacci ML, Forni M, Galeati G, Cerrito MG, Busnelli M, Lavitrano M, Giovannoni R. In vitro production of multigene transgenic blastocysts via sperm-mediated gene transfer allows rapid screening of constructs to be used in xenotransplantation experiments. Transplant Proc. 2010 Jul-Aug;42(6):2142-5.
25. Wagner EF, Stewart TA, Mintz B. The human beta-globin gene and a functional viral thymidine kinase gene in developing mice. Proc Natl Acad Sci U S A. 1981 Aug;78(8):5016-20.
26. Wall RJ. New gene transfer methods. Theriogenology. 2002 57, 189–201.
27. Wang L, Fan J, Yu M, Zheng S, Zhao Y. Association of goat (Capra hircus) CD4 gene exon 6 polymorphisms with ability of sperm internalizing exogenous DNA. Mol Biol Rep. 2011 Mar;38(3):1621-8. Epub 2010 Sep 17.
28. Zani M, Lavitrano M, French D, Lulli V, Maione B, Sperandio S, Spadafora C. The mechanism of binding of exogenous DNA to sperm cells: factors controlling the DNA uptake. Exp. Cell Res. 1995 217, 57–64.
29. Zhao Y, Yu M, Wang L, Li Y, Fan J, Yang Q, Jin Y. Spontaneous uptake of exogenous DNA by goat spermatozoa and selection of donor bucks for sperm-mediated gene transfer. Mol Biol Rep. 2011 Jun 12. [Epub ahead of print]



Drosophila melanogaster



Part 2 – Silencing myc through *RNA interference*

Introduction

Inhibition of protein synthesis extends lifespan in invertebrates and mammals. Lifespan extension has been obtained by knocking-down genes encoding ribosomal proteins or controlling translation initiation, by inhibiting the TOR pathway's activity, and by inhibiting ribosomal biogenesis through the use of drugs.

The TOR pathway is a central pathway in nutrient sensing conserved from yeast to humans (Evans et al., 2010), (Stanfel et al., 2009), (Wullschleger et al., 2006) it responds to amino-acid availability and to changes in the ATP/AMP ratio. Nutrient sensing in multicellular organisms is conveyed through the insulin/IGF pathway by the hormonal action of insulin. The insulin/IGF pathway triggers a cascade of phosphorylation events and, through the action of the PI3K and Akt kinases, activates the pathway branch containing the TOR complex 1 (TORC1). In the presence of an abundance of nutrients, TORC1 coordinates protein synthesis by promoting both ribosomal biogenesis and by regulating translation initiation via genes such as S6k and 4E-BP.

When nutritional resources are low or the animal is subject to stresses such as hypoxia or DNA damage, TOR activity is repressed to inhibit cell growth and proliferation. Consistently, inhibition of TOR activity has been observed in Dietary Restriction (DR) animals (Stanfel et al., 2009). In particular, epistatic studies in invertebrates are consistent with the hypothesis that TOR mediates at least part of the longevity response of DR (Hansen et al., 2007), (Kaeberlein et al., 2005), (Kapahi et al., 2004), (Hansen et al., 2008). In mammals, a recent study on DR mice has reported a significant increase in the expression of DNA-damage inducible transcript 4 (Ddit4), a key inhibitor of the TOR pathway, and a reduction in 4E-BP1 phosphorylation (Estep et al., 2009). Phosphorylated 4E-BP1 releases eIF4E, which is then free to associate with eIF4G to stimulate translation initiation (Wullschleger et al., 2006).

Interestingly, Zid et al (2009) show that: the d4E-BP protein, the sole fly 4E-BP ortholog, is upregulated in DR fruit flies; 4E-BP is required for maximal lifespan extension upon DR in Drosophila; and overexpression of activated d4E-BP extends lifespan. However, life span extension was abrogated in the activated 4E-BP alleles upon DR, consistent with a model in which DR increases levels of 4E-BP but at the same time, reduces 4E-BP phosphorylation.

Direct inhibition of TOR signaling has been shown to extend life span in yeast (Kaeberlein et al., 2005), worms (Vellai et al., 2003), and flies (Kapahi et al., 2004). Recently, several studies have shown that TOR activity modulates mammalian aging as well. Harrison et al. report that when genetically heterogeneous mice are fed the TOR inhibitor rapamycin, they live significantly longer than mice on a normal diet (28% for males and 38% for females increased life expectancy from time of treatment) (Harrison et al., 2009). Notably, these results were obtained by starting the rapamycin treatment at a relatively old age (20 months old mice, roughly equivalent to 60 years old humans), and similar results were obtained independently in three independent groups of mice at three research sites. Chen et al. describe a significant increase in mouse life span when rapamycin is fed to 22-24 month old mice, and report a protective effect from experimental infection with influenza virus when rapamycin is administered in a 6 week bout to even older mice (26 month) (Chen et al., 2009). In addition, life span extension and resistance to age-related pathologies has also been achieved in mice by directly targeting components of the TOR pathway, specifically by deleting the ribosomal S6 protein kinase 1 (S6K1) (Selman et al., 2009). The same study showed that S6K1 deletion induced expression patterns similar to those observed in DR.

Increased life span can also be achieved by modulating the expression of genes downstream of the TOR pathway such as genes involved in ribosomal biogenesis and translation initiation factors. For example, Steffen et al showed that deletion of genes encoding proteins within the 60S subunit or its processing factors extend lifespan in Saccaromyces cerevisiae, and that an analogous effect can be achieved by using a small molecule that inhibits the

60S subunit biogenesis (Steffen et al., 2008). In Caenorhabditis elegans life span extension has been achieved by inhibiting the gene encoding for ifg-1, the worm homologue of translation initiation factor eIF4G, which is a scaffold protein in the cap-binding complex (Pan et al., 2007).

Myc and protein synthesis

Myc encodes a transcriptional regulator whose inappropriate expression is correlated with a wide array of cancers (Dang et al., 2006), (Grandori et al., 2000), (Meyer et al., 2008). At the cellular level Myc activity has been linked with cell division, accumulation of mass, differentiation, and programmed cell death.

In the recent years Myc's central role in the regulation of protein synthesis has emerged (van Riggelen et al., 2010). Myc has been shown to regulate protein synthesis at multiple levels by controlling the transcription of ribosomal RNA and proteins, genes involved in processing of ribosomal RNA, genes involved in the nuclear export of ribosomal subunits, and translation initiation factors.

The Myc:Max:Mxd transcriptional network controls the transcription of a large fraction of the genome (at least 15%). Myc and Max form a heterodimer that recognizes and binds to E-box sequences found in the promoters and 5'UTRs of many genes. This binding recruits co-regulatory factors leading to the acetylation of nucleosomal histones and expression of the target gene. Max can form a heterodimer with members of the Mxd family, and such dimer can recognize and bind to E-boxes, and antagonize the Myc-Max complex activity. The Myc-Max heterodimer can also bind to the transcription factor MIZ1 and act as a transrepressor of MIZ1 targets. Finally, Myc can form a complex with an RNA polymerase III-specific transcription factor (TFIIIB) and stimulate the transcription of transfer RNA (tRNA) and 5S ribosomal RNA genes.

The Myc:Max:Mxd network in Drosophila has the advantage that it contains one member for each class: Myc, Max and Mnt. Hence it has a much simpler form than in higher organisms where each class can contain multiple homologs.

Teleman et al. have recently demonstrated that a link exists between the TOR pathway and Myc (Teleman et al., 2008). Specifically they showed that in D. melanogaster TORC1 appears to regulate Myc primarily at the post-transcriptional level, and that, in vivo, Myc acts as a mediator of nutrient-regulated gene expression downstream of TOR. Because the vast majority of these genes are involved in ribosome assembly, they propose a model in which Myc mediates TOR's regulation on ribosomal biogenesis.

Professor Neretti together with the Stephen Helfand's lab then performed a statistical analysis using a linear model to detect which genes 1) were significantly different between HC and LC and 2) switched from the HC level to the LC level as a consequence of the switch in diet. Using a False Discovery Rate (FDR) (Storey et al., 2003) of 10%, we detected 387 switching genes, which we then used to perform a pathway analysis to detect what functional categories were significantly enriched among them. For this purpose, we ran the list of genes on the FlyMine website which performs an enrichment analysis using several collections of biological pathways in Drosophila from multiple databases (Lyne et al., 2007). The top 14 functional pathways in the enrichment analysis of the KEGG and Reactome databases were related to translation, apart from a single one, which was related to insulin synthesis and secretion. Of the 387 genes identified, 63 were part of the "translation" gene ontology (GO:0006412) (Table 1), which was significantly enriched (hypergeometric test, $p<2x10-17$ after correction for multiple testing). As Table 1 shows, the list contains a large number of ribosomal proteins, whose expression is repressed after the switch and in DR flies.

Table 1 - Switch Genes in the "translation" Gene Ontology

Aats-asn	eIF-3p66	mRpL39	RpL18	RpL31	RpL40	RpLP1	RpS18	RpS3A
Aats-tyr	eIF4AIII	Qm	RpL18A	RpL32	RpL5	RpLP2	RpS19a	RpS4
Aats-val	eIF-4B	RpL10Ab	RpL19	RpL34b	RpL6	RpS11	RpS23	RpS5a
CG33158	eIF-5A	RpL11	RpL23	RpL35	RpL7	RpS13	RpS25	RpS8
CG4699	eIF6	RpL14	RpL23A	RpL35A	RpL7A	RpS15	RpS28b	RpS9
CG7414	mRpL3	RpL15	RpL26	RpL36	RpL8	RpS16	RpS29	Sta
Ef2b	mRpL38	RpL17	RpL27A	RpL37A	RpL9	RpS17	RpS30	Su(var)3-9

The Helfand lab performed several diet switch experiments and found a focused group of genes related to translation. Because of the well established role of protein synthesis in aging and the recent results linking Myc activity to ribosomal biogenesis they hypothesized that the Myc:Max:Mnt network activity could modulate lifespan. To test this, they decreased and increased Myc activity. A decreased activity can be achieved by either reducing Myc expression or by overexpressing its antagonist Mnt. According to literature, both interventions should lead to an increase in ribosomal biogenesis and translational activity. The opposite effect, that is a decrease in translational activity, can be achieved by reducing Myc expression.

One of the most informative experimental strategies to determine the function of a protein is to remove it from a biological system in which it normally functions and study the resulting effects.

Historically, loss-of-function analysis has been the domain of mutational genetics in model organisms. Over the past few years, however, double-stranded RNA-induced gene silencing (RNAi) has emerged as a powerful tool for the functional characterization of proteins at the cellular level. In essence, RNAi blocks production of a specific protein and induces loss-of-function phenotypes as protein levels drop due to protein turnover and serial dilution by cell division (Fire et al., 1998), (Elbashir et al., 2001). Cultured Drosophila cell lines have become well-known experimental systems to use in combination with RNAi for both biological and technical reasons. Many different fly cell lines that were derived from various tissues or developmental stages are available from the Drosophila Genome Resource Center in Bloomington, IN (https://dgrc.cgb.indiana.edu/). Although some of the available lines are uncharacterized, many of them (e.g., S2, S2R+, cl-8, Kc167) are highly susceptible to RNAi after treatment with large double-stranded RNAs (dsRNAs). This is an advantage fly cells have over mammalian cells: use of longer dsRNAs eliminates the cost associated with siRNA synthesis necessary for mammalian cells and circumvents the difficulties associated with siRNA design. In addition, the Drosophila genome exhibits less

functional redundancy compared with mammalian systems, which makes it easier to produce a loss-of-function phenotype by inhibiting a single gene at a time. Finally, with the development of commercially available dsRNA libraries and screening facilities, fly cell lines have been used very effectively as gene discovery tools for a variety of cellular processes (Echeverri et al., 2006).

The most widely used Drosophila cell line is named S2. S2 cells were originally derived from late embryonic stage Drosophila embryos as a spontaneously immortalized, nonclonal cell line (Schneider et al., 1972). These cells exhibit mesodermal characteristics and their repertoire of cellular behaviors and gene expression suggest that they are derived from hemocytes—professional macrophages that, in the fly, are responsible for phagocytosis of invading bacteria and apoptotic cells (Ramet et al., 2002). Morphologically, S2 cells come in at least two different varieties. The parent S2 cell line is roughly spherical, 15–20 mm in diameter, and adheres rather weakly to most tissue culture substrates. A second sub-line of S2 cells, designated S2R+, was isolated based on its ability to respond to application of soluble wingless protein and was conferred with the 'R+' designation to reflect its different complement of membrane receptors. S2 and S2R+ cells divide rapidly, with about a 20-h cell cycle at room temperature (20–25 °C), and do not require specialized equipment for their culture apart from a sterile hood for passage. Thus, use of this cell line has many advantages to use as a model to study basic cellular function.

Aim

The area of interest of this project is to study in detail the sub-pathway which includes Myc's control of protein synthesis via regulation of ribosomal biogenesis and translation initiation factors, and its effect on longevity.

Therefore, in particular the aim is to develop and validate an optimal model in vivo and in vitro for Myc inhibition. The goal of this aim is to identify a translational and a transcriptional signature for lifespan extension via Myc through the protein synthesis pathway. The set of genes comprising these signatures will tell us which and how specific pathways are affected by Myc-induced inhibition of protein synthesis, and will help us understand how these changes can have a lifespan extending effect.

Material and methods

Cell culture

We obtained *Drosophila melanogaster* Schneider 2 (S2) cells from the Drosophila Genomics Resource Center (DGRC) and used standard protocols for both cell culture and RNA interference (RNAi) as described previously by Rogers SL and Rogers GC (2008).

The S2 cell line is derived from late embryonic stage *Drosophila* embryos as spontaneously immortalized, nonclonal cell line (Schneider et al., 1972). The optimal culture condition in a incubator is 25°C with atmospheric CO_2 and O_2 concentration.

The growth medium, Sf-900 II SFM (Invitrogen, cat. No. 10902), was brought to room temperature and then 1 ml of medium was dispensed per well in a 6-well plate (Costar). Next, frozen cells were thawed by immersing the vial in a 37°C water bath for less than a minute, then the cell suspension (1.2 ml) was transferred in the wells (200 µl each) using a sterile pipette in a cell culture hood. Cells were allowed to attach the bottom of the plate for 1h then the medium was gently removed and replaced with an equal volume of Sf-900 II SFM in order to avoid prolonged exposure to DMSO (present in the long-term storage frozen vials). To recover from thawing cells need 5-6 days of growth at 25°C: then they should be in optimal condition to start passaging.

Starting from the early passages, cells were split every 3-4 days at a constant ratio of 1:5.

RNAi target genes

Five genes were knocked down through RNAi; the information about the chosen genes are found on FlyMine (www.flymine.org) and in numerous papers (Teleman et al., 2008).

Gene	**qm** *D. melanogaster*
NCBI Gene Number	38816
Name	quemao
Pathways	Metabolic - Terpenoid backbone biosynthesis
Length	5308

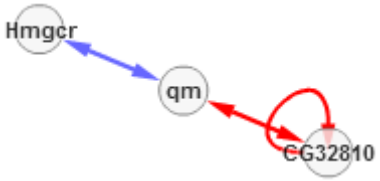


Figure 3: Qm mapped interactions, both genetic (blue) and physics (red)

Gene	**RpLp0** *D. melanogaster*
NCBI Gene Number	40451
Name	Ribosomal protein LP0
Pathways	Ribosome - Eukaryotic Translation Termination - 3' UTR mediated translational regulation
Length	1446

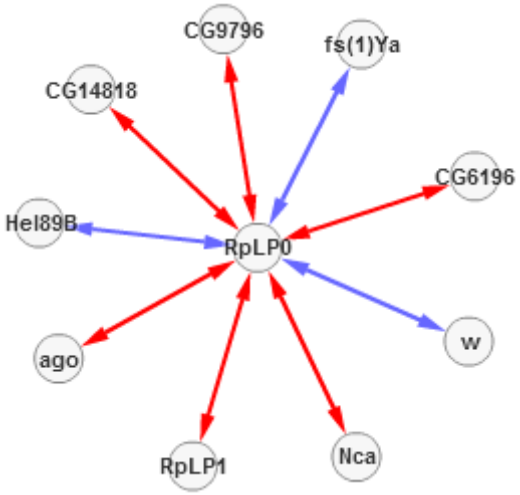


Figure 4: RpLp0 mapped interactions, both genetic (blue) and physics (red)

Gene	**RpLp2** *D. melanogaster*
NCBI Gene Number	36855
Name	Ribosomal protein LP2
Pathways	Ribosome - Eukaryotic Translation Termination - 3' UTR mediated translational regulation
Length	639

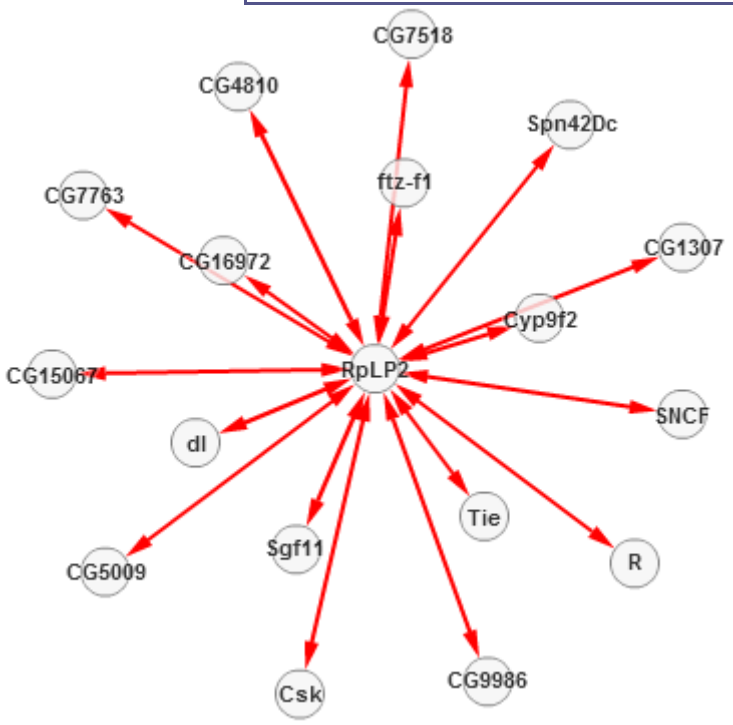


Figure 5: RpLp2 mapped interactions, no genetic interactions are visible (blue), only physics (red)

Gene	**dm** *D. melanogaster*
NCBI Gene Number	31310
Name	Diminutive
Pathways	Cell cycle, Mitotic
Length	12834

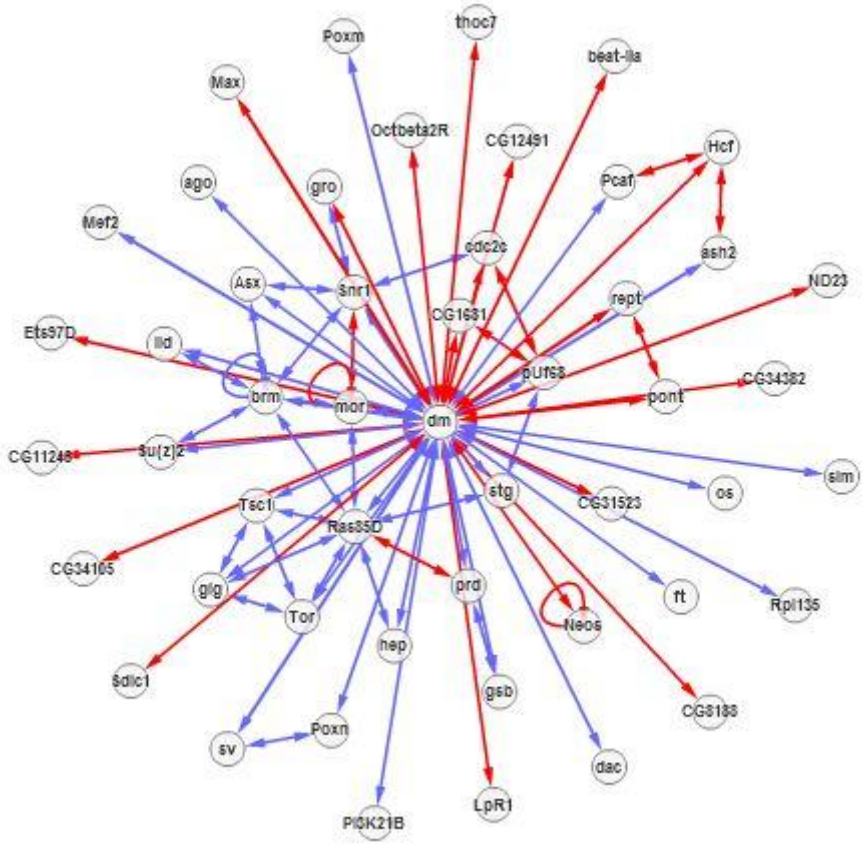


Figure 6: dm (myc) mapped interactions, both genetic (blue) and physics (red)

Gene	**RpS3** *D. melanogaster*
NCBI Gene Number	42761
Name	Ribosomal Protein S3
Pathways	Ribosome - Eukaryotic Translation Termination – 3' UTR mediated translational regulation
Length	12834

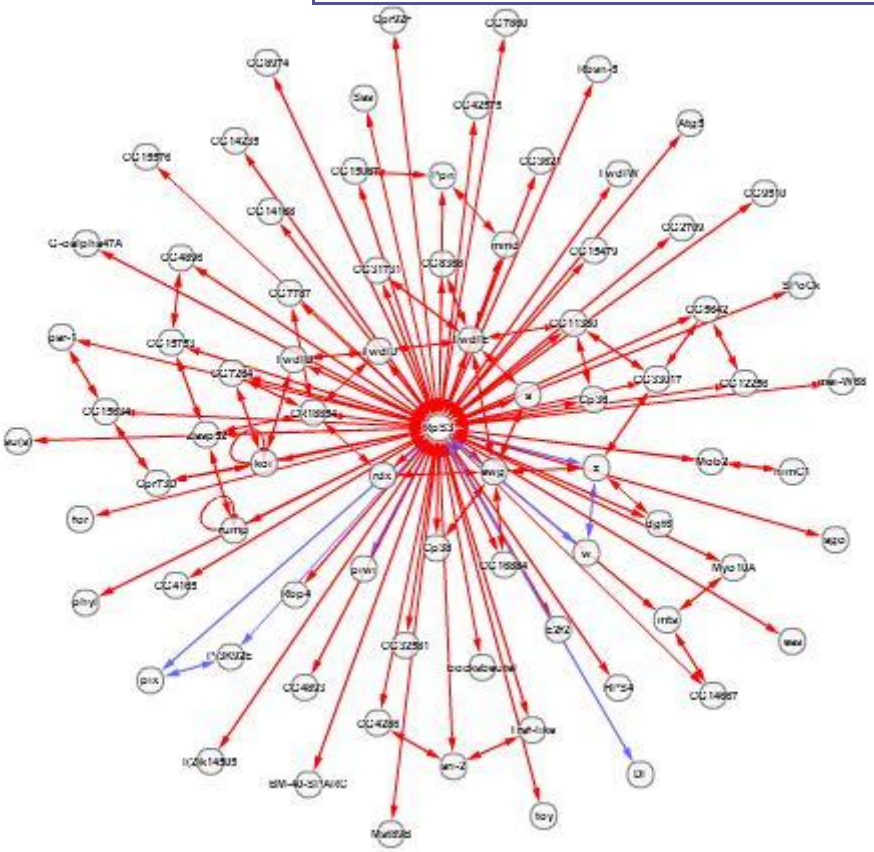


Figure 7: RpS3 mapped interactions, both genetic (blue) and physics (red)

As suggested by Rogers et al., a positive control for RNAi was included in all the steps of cell culture. The Rho1 gene was picked because its efficient inhibition prevents the cells from completing cytokinesis, so the diameter increases two-three times.

Gene	**Rho1** *D. melanogaster*
NCBI Gene Number	36775
Name	Rho1
Pathways	Several but in this case, focus on cytokinesis
Length	12834

dsRNA production

Genomic DNA was used to amplify via PCR the target genes. Each primer contained the sequence of T7 promoter as described previously by Rogers et al.

For all the target genes two regions were selected to be part of the experiments, with the exception of RpLp2 that has only one: as a matter of fact according to literature that is the only possible target for an effective *interference* experiment.

ID	R primer w/ T7	S primer w/ T7
dm (pair 1)	*TAATACGACTCACTATAGG* AGAGATCCGCAACATCGACT	*TAATACGACTCACTATAGG* CGCTTCTGACAGACCGTGTA
dm (pair 2)	*TAATACGACTCACTATAGG* CTACCCACAAATCCCTCGTG	*TAATACGACTCACTATAGG* TCTGATACGGTGTGCTCGAC
RpS3 (pair 1)	*TAATACGACTCACTATAGG* GTTTCAACTTCGAGACCGGA	*TAATACGACTCACTATAGG* ACGAATTTCATCGACTTGGC
RpS3 (pair 2)	*TAATACGACTCACTATAGG* AGGTCATGTTGCCCTACGAC	*TAATACGACTCACTATAGG* TCAGCTAGTCGCTCATTGGA
RpLPo (pair 1)	*TAATACGACTCACTATAGG* CGATGAGTTCCCAAAGTGCT	*TAATACGACTCACTATAGG* TGTTCCCTTGGAAATTTTGG
RpLPo (pair 2)	*TAATACGACTCACTATAGG* AACATCTCGCCCTTCTCGTA	*TAATACGACTCACTATAGG* TTCTTGAATCCGTTGGCAAT

RpLP2 (pair 1)	*TAATACGACTCACTATAGG* AATAAGCGGTTGAATGTGGC	*TAATACGACTCACTATAGG* AAATGGTGAACGGATGGGT
Qm (pair 1)	*TAATACGACTCACTATAGG* TGCAAAAATAAGCCGTACCC	*TAATACGACTCACTATAGG* CGCATTCTGATGTGGAACTG
Qm (pair 2)	*TAATACGACTCACTATAGG* CTTCAAACTGGAATGCGAGG	*TAATACGACTCACTATAGG* TTTTGACGTCCAGGGAACTT

Primers were resuspended in TE pH 8.0, in a stock concentration of 100 µM. Working aliquots for each primer pair consisted of a 1:10 dilution in water. End-point PCR were then setup according to Taq Platinum (Invitrogen) suggested protocol:

DNA template for dsRNA production	
Buffer 10X	5 µl
$MgCl_2$	3 µl
dNTPs (2.5 mM each)	5 µl
T7 containing primers	1 µl
DNA (50 ng)	1 µl
Taq Platinum	0.5 µl
sterile, deionized water	34.5 µl

An aliquot of the PCR products was run on a 1.5% agarose gel in TAE 1X for 60 minutes at 80 V constant. The remaining part of the PCR was purified with Qiaquick PCR Purification Kit (Qiagen), eluted in 35 µl H_2O and quantified with Nano-Drop spectrophotometer.

The T7 RNA polymerase promoter was used in the synthesis of dsRNA molecules with the T7 RiboMAX™ Express RNAi System (Promega).

T7 reaction components	
RiboMAX 2x buffer	20 µl
DNA (~ 1 µg) + Water	16µl
Enzyme mix	4 µl

Transcription reactions were incubated at 37°C overnight: the following day the dsRNA were made according to the manufacturer's protocol, briefly:

- Anneal the RNA strands by incubating at 70°C for 10 minutes, then slowly cool to room temperature in a thermocycler (~20 minutes): this allows annealing of the double stranded RNA
- Dilute the RNase Solution (supplied with the kit) 1:200 in Nuclease-Free Water
- Add 2 µl of the freshly diluted solution and 2 µl of RNase-Free DNase (supplied with the kit) to the reaction.
- Incubate for 30 minutes at 37°C: this removes template DNA and any remaining single-stranded RNA
- Add 4 µl of 3M of Sodium Acetate (pH 5.2) and 40 µl of isopropanol, mix well and incubate 5 minutes on ice.
- Spin a microcentrifuge at 13,400 rpm for 10 minutes
- Wash the pellet with 500 µl ice-cold 70% Ethanol
- Resuspend dsRNA pellets with 100 µl Nuclease-Free Water, quantify with Nano-Drop spectrophotometer and run a 1 µl aliquot on a 1.5% agarose gel in TAE 1x
- Split into aliquots in order to avoid freeze-thaw cycles and store at -80°C

RNAi treatment on S2 cells

The use of S2 cells for RNAi experiments was performed following the previously described procedure by Rogers et al. (Rogers et al., 2003), briefly:

- Plate the cells one day in advance, in order to start with a ~50% confluency
- For each RNAi treatment, pipette 1 ml of Sf-900 II SFM medium into a 1.5 ml tube and dilute the dsRNA into the medium to a concentration of 10 µg/ml
- Carefully remove the medium from cells (loosely attached to the bottom of the plate)
- Replace it with dsRNA-containing medium
- Repeat daily for 6 more days

Total RNA and protein extraction

Total RNA extraction from S2 cells was performed according to Trizol Reagent ® (Ambion) manual. Briefly:

- Harvest cells by centrifugation and remove the media: no washing steps were included, as suggested by the product manual, in order to avoid the chances of mRNA degradation
- Add 750 µl of TRIzol® Reagent per 250 µl of sample (generally ~10 x 10^6 cells)
- Lyse the cells in sample by pipetting up and down several times
- Incubate at room temperature for 5 minutes, to permit the complete dissociation of the nucleoprotein complex
- Add 200 µl of Chloroform
- Vortex vigorously for 15 seconds
- Incubate 3 minutes at room temperature on the bench
- Centrifuge the sample at 12,000 *x g* for 15 minutes at 4°C
- Transfer the aqueous phase of the sample (~500 µl) in a new tube
- *Keep the organic phase in the fume hood for later protein extraction*
- Add 500 µl (1 volume) of 100% isopropanol
- Incubate at room temperature for 10 minutes
- Centrifuge at 12,000 × *g* for 10 minutes at 4°C
- Remove the supernatant from the tube, leaving only the RNA pellet

- Wash the pellet with 1 mL of 75% ethanol
- Vortex the sample briefly, then centrifuge the tube at 7500 × g for 5 minutes at 4°C and discard the wash
- Air dry the RNA pellet for 2-3 minutes
- Resuspend in 100 µl RNase-free water

At this point, samples can either be stored at -80°C or purified for any non-RNA carryover with RNeasy ® Mini Kit (Qiagen). RNA Cleanup followed the manufacturer protocol, briefly:

- Add 350 µl Buffer RLT to the sample and mix well
- Add 250 µl ethanol 100% to the diluted RNA and mix well by pipetting: avoid vortexing
- Transfer the sample (700 µl) to a RNeasy Mini spin column. Spin for 15 seconds at 13,000 rpm and discard the flow-through
- Add 500 µl Buffer RPE to the RNeasy spin column. Let the column sit on the bench 5 minutes, then spin for 15 seconds 13,000 rpm and discard the flow-through
- Again, add 500 µl Buffer RPE to the RNeasy spin column. Let the column sit on the bench 5 minutes, then spin for 2 minutes at 13,000 rpm and throw away the 2 ml tube
- Place the RNeasy spin column in a new 1.5 ml eppendorf tube.
- Add 50 µl RNase-free water directly to the spin column membrane. Let the column sit on the bench 5 minutes, then spin for 2 minutes at 13,000 rpm.
- Repeat the elution of RNA with 50 µl RNase-free water in the same collection tube

Purified RNAs were then quantified with Nano-Drop spectro-photometer (Thermo Scientific) for micro-samples and stored at -80°C.

Spike production

A spike for RNA quantification was prepared digesting plasmid pLKO.1-Puro (Addgene plasmid 8453) with KpnI (Promega) as follows:

pLKO.1-Puro linearization reaction	
sterile, deionized water	15.3 µl
Optimal buffer – J – 10X	2 µl
Acetylated BSA, 10 µg/µl	0.2 µl
DNA (pLKO.1), 1 µg/µl	2 µl
Restriction Enzyme Kpn I, 10 U/µl	0.5 µl

Incubate for 3 hours at 37°C.

The linearized plasmids were purified with AMPure XP (Agencourt) magnetic beads:

- Add 36 µl of beads and mix well
- Let the beads bind to DNA leaving the tube on bench for 5 minutes
- Transfer the tube to a magnetic separator and wait until clear
- Aspire surnatant and wash the beads twice with 500 µl ethanol 70%
- Spin the beads briefly, transfer the tube again on the magnetic separator and aspirate the remaining traces of ethanol 70%
- Air dry for 1-2 minutes
- Add 16 µl Nuclease-free H_2O, mix well and incubate on bench for 5 minutes
- Put the tube on magnetic separator and transfer the solution, once clear, in a new tube

Finally a transcription reaction was setup with T7 RiboMAX ™ Express RNAi System as follows:

DNA + water	16 µl
RiboMAX 2X buffer	20 µl
Enzyme mix	4 µl

The reaction containing tube was incubated overnight at 37°C.

The following day, 1 µl of DNase (supplied with the kit) was added to the reaction and incubated at 37°C for 30 minutes, in order to digest the original DNA template. An isopropanol precipitation was carried over and the RNA spike purified and resuspended in 100 µl of Nuclease-free water. Finally the RNA was quantified with Nano-Drop spectrophotometer and an aliquot was run on 1.5% agarose gel.

Ribosomal RNA quantification

The purified total RNA was brought to a concentration of 250 ng/µl in water and run in a RNA nano chip with Bioanalyzer 2100 (Agilent). The resulting electropherograms were exported as raw lists of time and signal and the area underlying the ribosomal peaks (18S and 28S) was integrated. Next the experiment was repeated for the same samples with the addiction of the generated RNA spike (50 ng/µl final) and once again the area underlying the peaks (ribosome subunits and the spike) was integrated. Finally the ribosomal RNA content was normalized to the content of the spike and compared to the direct measure operated previously.

mRNA extraction

The mRNA extraction was carried out with the Dynabeads mRNA DIRECT kit (Invitrogen) according to the manufacturer's protocol with some adjustments. A total of 25 bodies or 25 heads was processed for an expected yield of, respectively, 250-350 ng and 25-50 ng of mRNA. It is very important to keep everything cold and work quickly because of the intrinsic fragility of the mRNAs. Because of this, no more than six samples were processed at a time.

- Homogenize tissue in 500µl Lysis/Binding buffer, using 2ml ground glass homogenizer
- Extract lysate from the homogenizer with a pipette and place on ice
- Spin at 13,400 rpm for 3 minutes at 4°C and transfer surnatant to a new 1.5 ml tube
- *During centrifugation* – take 100 µl beads into a eppendorf tube, place on a magnetic stand and remove buffer once clear. Wash with Lysis/Binding Buffer
- Pass lysate 5 times through a 21g needle, using a 1 ml syringe, to shear DNA
- Remove buffer from beads and add lysate
- Incubate on a tube rotator at room temperature for 5 minutes
- Capture beads for 2 minutes on the magnetic stand
- Wash beads twice in 200 µl Wash Buffer A (supplied with the kit)
- Wash beads twice in 200 µl Wash Buffer B (supplied with the kit)
- Spin briefly at low speed, transfer on magnetic stand and suck out any lasting drop
- Resuspend in 20 µl Tris-HCl elution buffer and place at 65 °C for 2 minutes
- Capture beads on magnet immediately, transfer mRNA containing supernatant in a new tube and put on ice

The extraction was repeated so to cut down on rRNA contamination, re-using the same beads. Before proceeding to the second extraction, beads were washed twice in Wash Buffer B. Dilute the eluted mRNA five times in Lysis/Binding Buffer (100 µl final), and repeat the procedure. Elute again the mRNA in 20 µl Tris-HCl, quantify and store at -80 °C

RT-PCR

The reverse transcription (RT) and polymerase chain reaction (PCR) amplification was performed with the TaqMan Reverse Transcription Reagents (Invitrogen): based on a MultiScribe® Reverse Transcriptase, a

recombinant Moloney Murine Leukemia Virus Reverse Transcriptase, and the combination of random hexamers and oligo $(dT)_{16}$, the kit allows the possibility to target either total RNA or mRNA.

The amount of RNA used for each reaction was constant: 1.5 µg when starting from total RNA and 50 ng for mRNA.

RT reaction	
Buffer 10X	5 µl
$MgCl_2$	11 µl
dNTPs	10 µl
oligo $(dT)_{16}$	1.25 µl
Random hexamers	1.25 µl
RNase Inhibitors	1 µl
MultiScribe® RTase	1.25 µl
RNA + H_2O	to 50 µl

The reverse transcription thermal cycling was always the same: 10 minutes at 25 °C, 30 minutes at 48°C, inactivation with 5 minutes at 95 °C then hold at 4 °C. After thermal cycling, all cDNA samples were stored at -20 °C.

qPCR - targets

Five more target genes beyond the previously described were included in the qPCR detection

Gene Symbol	CG number	Flybase link
Nop60B	CG3333	http://flybase.org/reports/FBgn0259937.html
PPAN	CG5786	http://flybase.org/reports/FBgn0010770.html
Act5C	CG4027	http://flybase.org/reports/FBgn0000042.html
4E-BP	CG8846	http://flybase.org/reports/FBgn0261560.html
eIF4E	CG4035	http://flybase.org/reports/FBgn0015218.html

qPCR - primers

All primers were designed with Primer Express Software Version 3.0 (Applied Biosystems)

Gene Symbol	forward primer	reverse primer
Dm	CAGTTCCAGTTCGCAGTCAA	TTGACTCCCGGCAGATAAAC
RpS3	CATTGAGTTGTACGCCGAGA	CTCCGGTGAGCTTGTACCTC
RpLP0	AGGCGACACTGCTCAACATG	GGAGTCGTAGACCTGGTTGACA
RpLP2	CAGCTCTGTGGGCGTTGAG	ATCAGGTCGTCGATGCTCTTG
Qm	GTGGGGATTCACCAAATACG	CTCTGGGCGGTATTTCACAT
Nop60B	TCGTCATTTGCACCACCAA	GCCATAGTGGCTGTGGTCATG
PPAN	CGAGGAACCAGACGAAGAACTG	TTCCGCCGCCCAAACTA
Act5C	CGTGAGAAGATGACCCAGATCA	AGCCTGGATGGCCACATACA
4E-BP	CGAGCGGGCTTTCATGAA	CGTTGGACGGCGGAGTT
S6k	AGACCATTCTGAAAGCCAAGCT	CAGGCGACGCACCAGATC
Mnt	ATTGGCGGCGTGATCTATG	GCTCCACATGCCGAGGAT
eIF4E	CCCGCCGAGGCTAAGG	CAGTGTTGCCTGCTGGTTCA

Primers for qPCR were resuspended in TE pH 8.0 with a stock concentration of 110 µM. Working aliquots were made out of a ten fold dilution in H_2O (11 µM final) and stored at -20 °C.

qPCR - reactions

All the qPCR reactions were carried on MicroAmp® Optical 96-Well reaction plates (Applied Biosystems), with SYBR Green PCR Master Mix® (Applied Biosystems) in a ABI PRISM® 7900HT (Applied Biosystems) Sequence Detection System.

qPCR reaction mix	
SYBR Green mix 2x	10 µl
Primers	1 µl
cDNA	1 µl
H_2O	8 µl

Polysome gradient - Stock solutions

This technique relies on the absence of RNase throughout the all process; therefore the use of clean glass bottles and sterile equipment during the preparation of the stock solutions has a crucial role. Moreover all the solutions have been filter-sterilized before being stored at 4 °C.

10X Polysome Salts Stock	
Tris-HCl [pH 7.4]	150 mM
$MgCl_2$	100 mM
KCl	2.0 M
ddH_2O	To 500 ml

55% Sucrose Stock	
Sucrose	550 g
10X Polysome Salts Stock	100 ml
ddH_2O	To 1000 ml

10% Sucrose Stock	
Sucrose 55%	91 ml
10X Polysome Salts Stock	41 ml
KCl 3.0 M	16.67 ml
ddH_2O	To 4ml

45% Sucrose Stock	
Sucrose 55%	245 ml
10X Polysome Salts Stock	5.46 ml
KCl 3.0 M	10 ml
ddH_2O	To 297 ml

Lysis Buffer	
Tris-HCl [pH 7.4]	15 mM
$MgCl_2$	15 mM
KCl	350 mM
Triton X-100	1 %
RNase Inhibitor	40U/ml
ddH_2O	To 297 ml

Gradient Buffer	
Tris-HCl [pH 7.4]	15 mM
$MgCl_2$	15 mM
KCl	300 mM

A 10 mg/ml stock solution of cycloheximide in PBS was prepared on the day of use and diluted 1:100 in the following solutions:

- Cell culture media
- Lysis buffer
- PBS wash buffer
- 10% sucrose
- 45% sucrose

Polysome gradients - protocol

The experimental protocol was kindly supplied by Mike Bronson, PhD and later described in literature (Bronson et al., 2010).

- Mark the half volume line in a 15ml polyallomer tube (Beckman)
- Pipette ~7 ml of 10% sucrose, then with a cannula carefully underlay it with 45% sucrose to the marked half volume line
- Prepare a continuous 10-45% linear sucrose gradient with a Gradient Master (BioComp Instruments) and cool them at 4°C
- Wash the plates to harvest twice with ice-cold PBS (added with cycloheximide), dry well and quickly
- Add 200 µl lysis buffer to each plate and scrape down cells
- Transfer in a 1.5 ml tube
- Place the tube onto a tube rotator for 10 minutes
- Pass lysate 5 times through a 26g needle, using a 1 ml syringe, to break nuclei
- Pellet nuclei by spinning at 13,400 rpm for 12 minutes at 4°C and transfer surnatant into a new tube
- Determine the OD260 and ABS 260/280 ratio of the extract by adding 2 µl of extract to 198 µl ddH_2O with a spectrophotometer measurement
- Add dropwise the lysate to the gradients, adjusting paired samples for measured OD
- Ultra-centrifuge in a SW40 Ti rotor (Beckman Coulter) for 3 hours at 36,000 rpm at 4°C
- Carefully remove the tubes from the rotor and proceed with the analysis
- Polysome profiles were collected with a gradient fractionator (Brandel) and determined by monitoring RNA absorbance at 254 nm: the UV monitor sensitivity level was finally adjusted according to the loaded ODs

Results

Cell culture - RNAi treatment

To quickly assess the efficiency of RNAi silencing on S2 cells a 7-days experiment was carried over using dsRNAs for Rho1and dm, beyond Not Treated (NT) cells. Inhibition of Rho1 using RNAi produces a clearly recognizable change in S2 cells. (Fig 6 and 7) S2 cells were treated with control (SK) dsRNA for 7d and photographed through phase-contrast microscopy. Cells are typically 5–10 mm in diameter. (Fig 7) In contrast, cells treated with dsRNA to deplete Rho1 for the same amount of time fail to complete cytokinesis and increase twofold to fourfold in diameter.

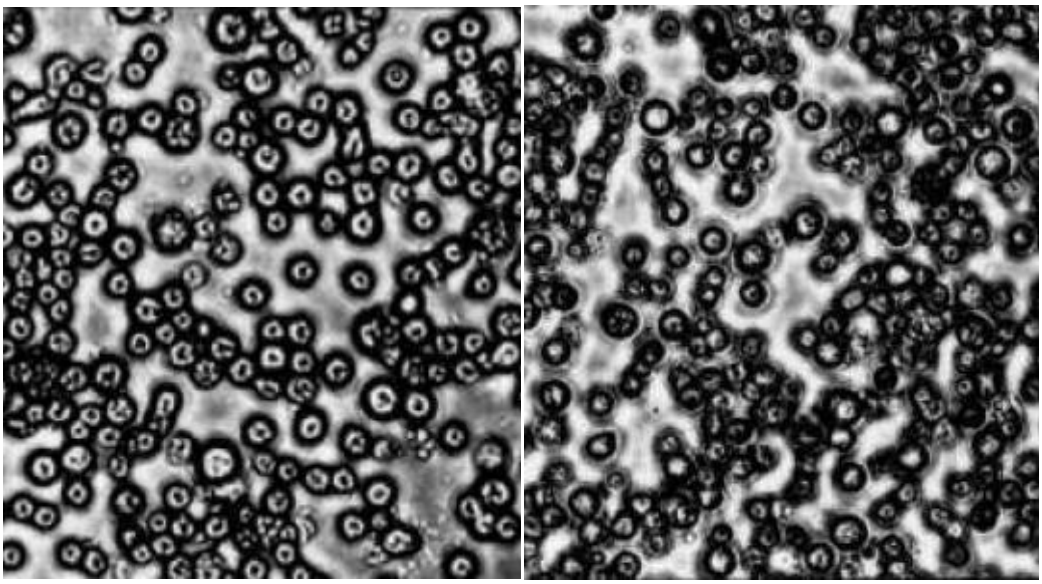


Figure 8: Not Treated S2 cells after 2 days (on the left) and 7 days (on the right)

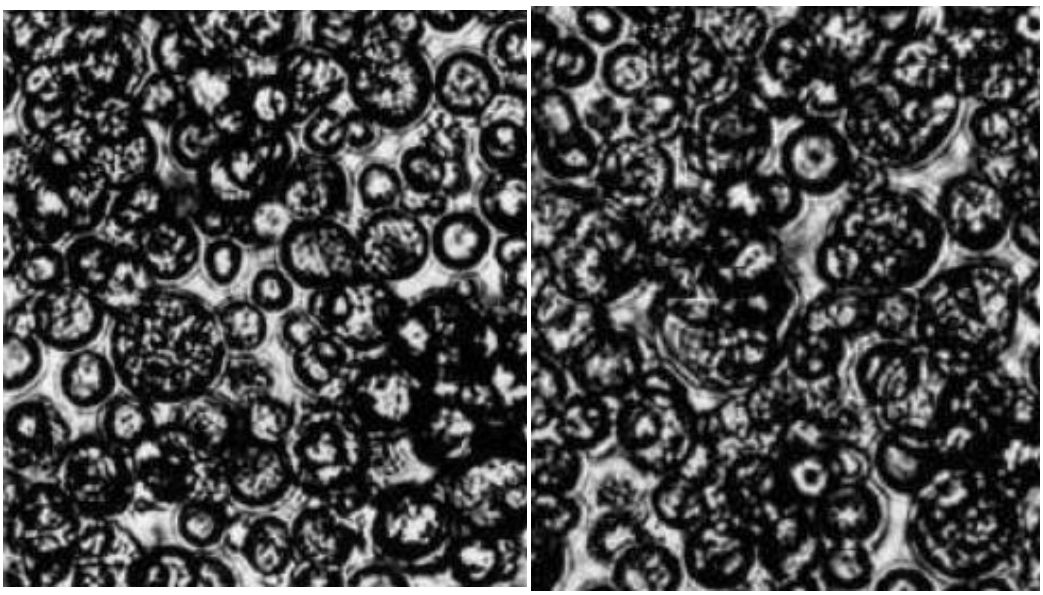


Figure 9: Rho1 S2 cells after 2 days (on the left) and 7 days (on the right)

At the end of the seventh day of treatment, a 1:1 dilution of cells was counted and measured in viability and diameter with a Countess Automated Cell Counter ® (Invitrogen)

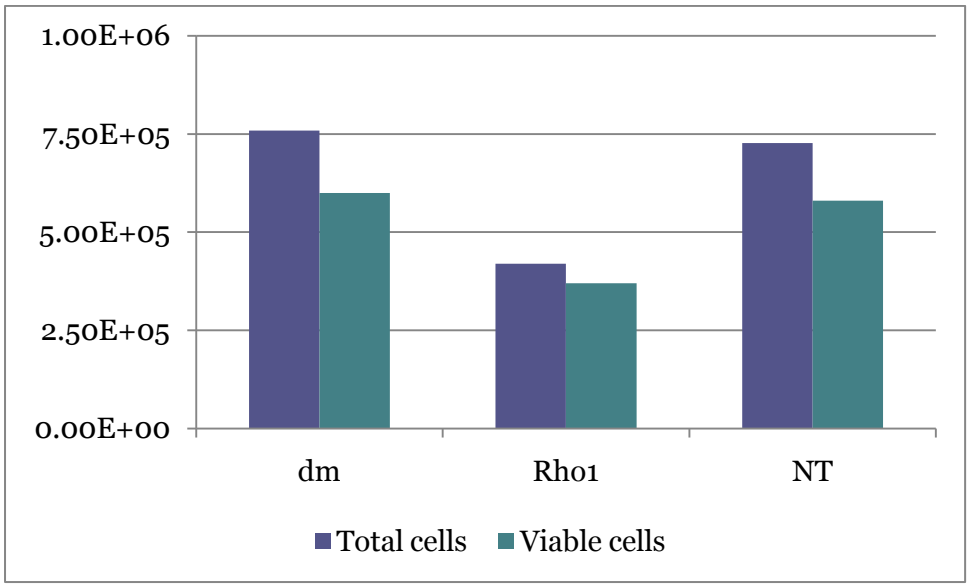


Figure 10: preliminary RNAi treatment

The difference in size is statistically different among all the samples after a t test: Rho1 cells are bigger than the other ($p < 0.01$) and dm cells are smaller than not treated ones ($p < 0.05$)

	Average diameter (mm)	Standard Dev
Rho1	27.99	1.5
NT	9.80	0.23
dm	9.29	0.22

Then the experiment was extended to all the produced dsRNA: dm, Qm, RpLp0, RpLp2 and RpS3. After 48 hours and 144 hours of treatment the cells were counted and measured in viability.

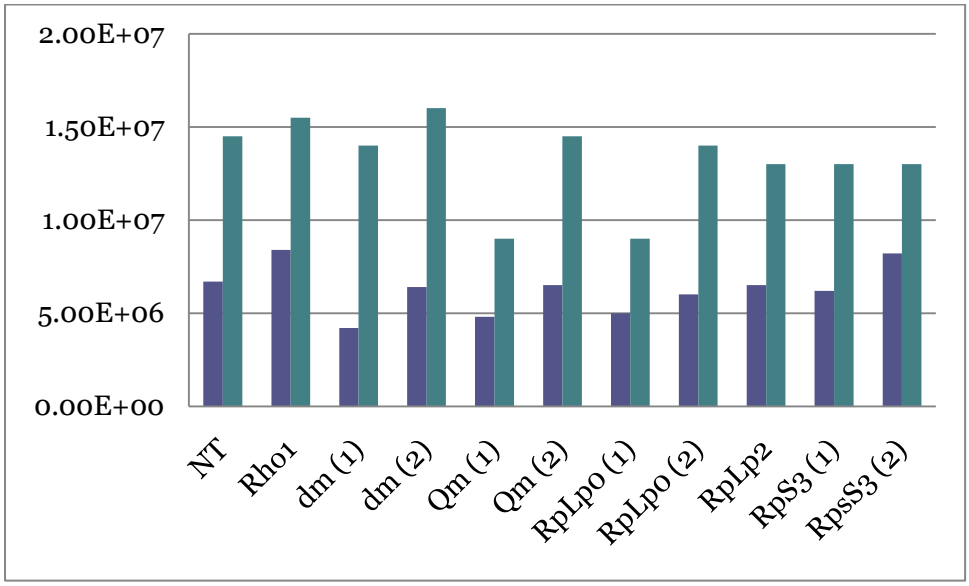


Figure 11: total cells

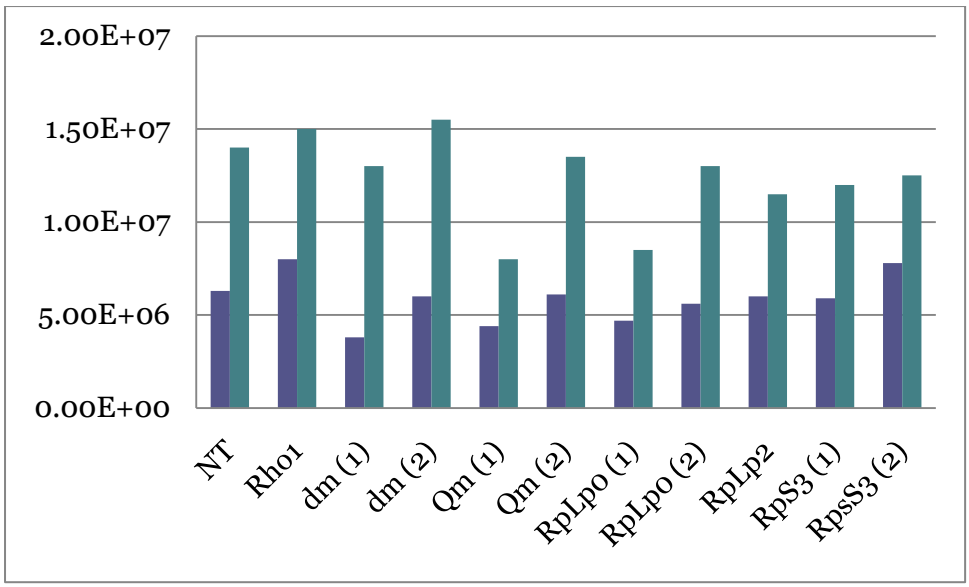


Figure 12: viable cells

Depending on the dsRNA in use the cell proliferation might be dramatically affected: it is the case of Qm and RpLp0, they both show a difference of nearly 50% in cell abundance between their vector (1) and (2) (Fig 2-3). The rest of the genes (dm and RpS3) showed no difference at all as resulting effect of the two variants of dsRNA.

Interestingly, the toxicity of dsRNA does correlate in a symmetrical way with the proliferation data (Fig 4): a sharp difference is present in RpS3 where the mortality almost doubled between the two variants, as the same stands for dm.

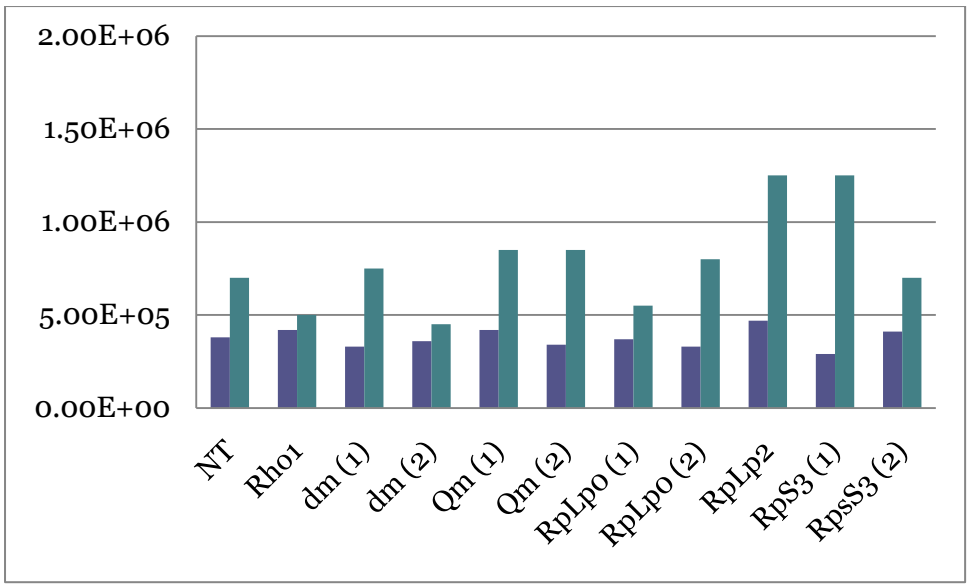


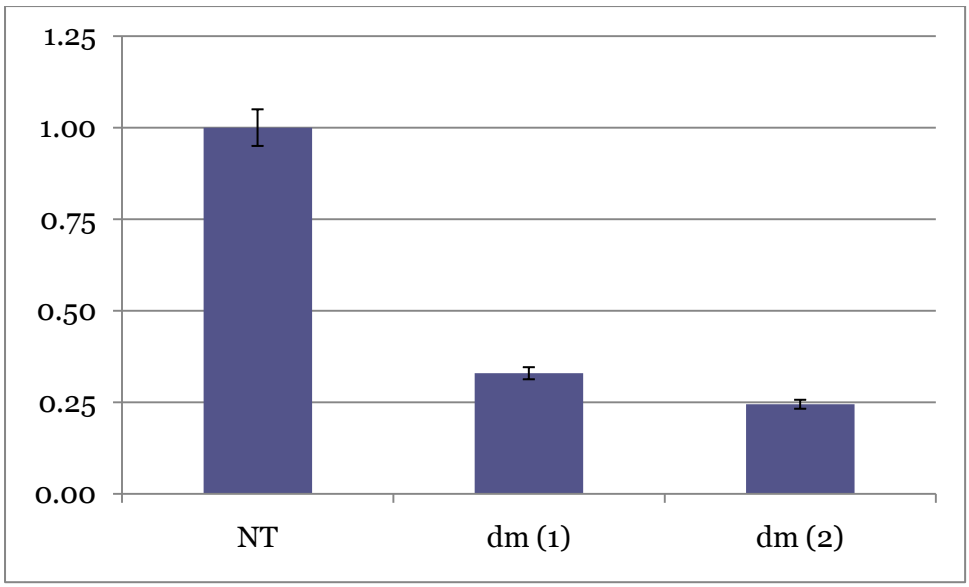
Figure 13: dead cells

At the end of the 7-days treatment cells were either processed for RNA extraction or translational analysis via polysome gradients.

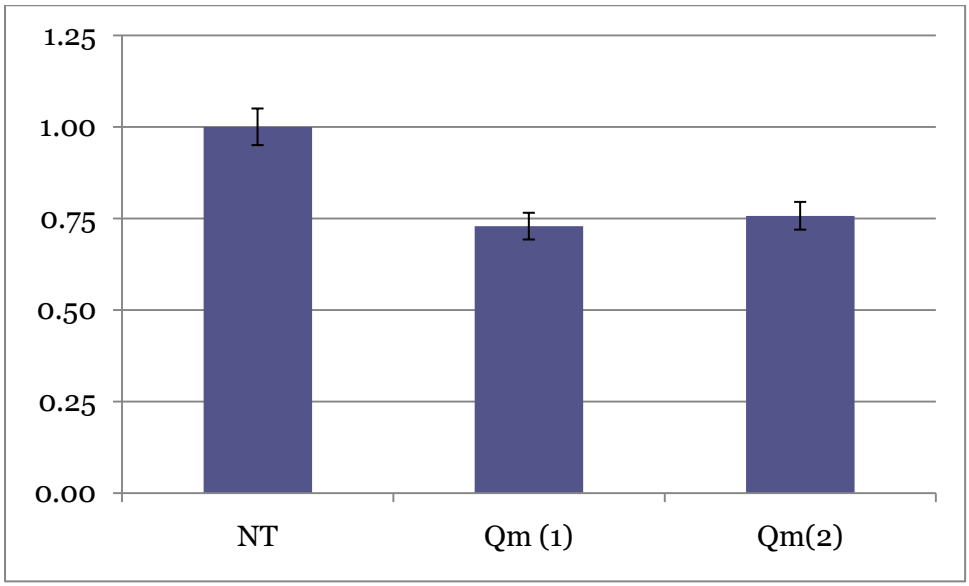
qPCR - S2 cells

The cDNA produced after the RNAi treatment from the S2 cells was first investigated for effective reduction in the target transcript.

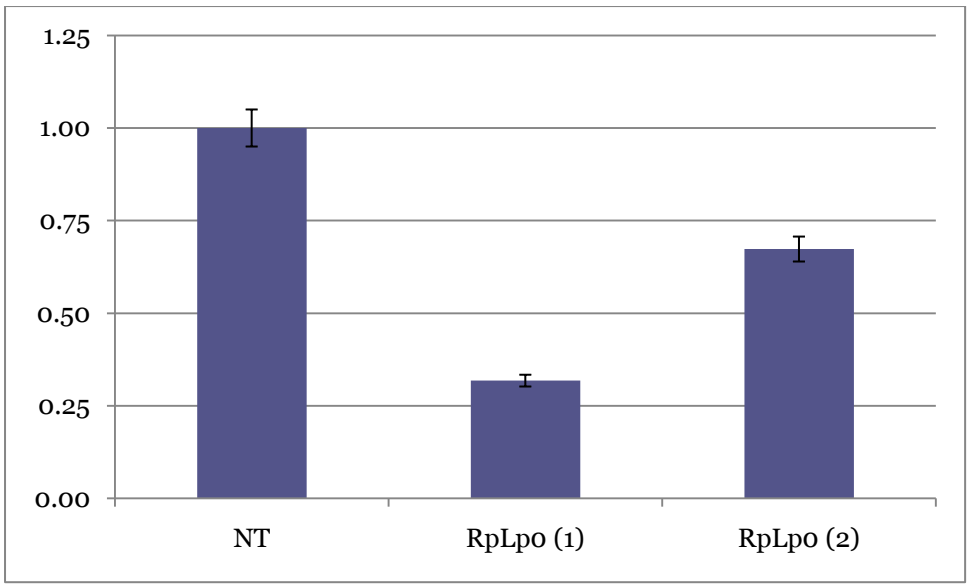
Both dm(1) and dm(2) strongly affected the presence of the target transcripts for an overall decrease of almost 75%.



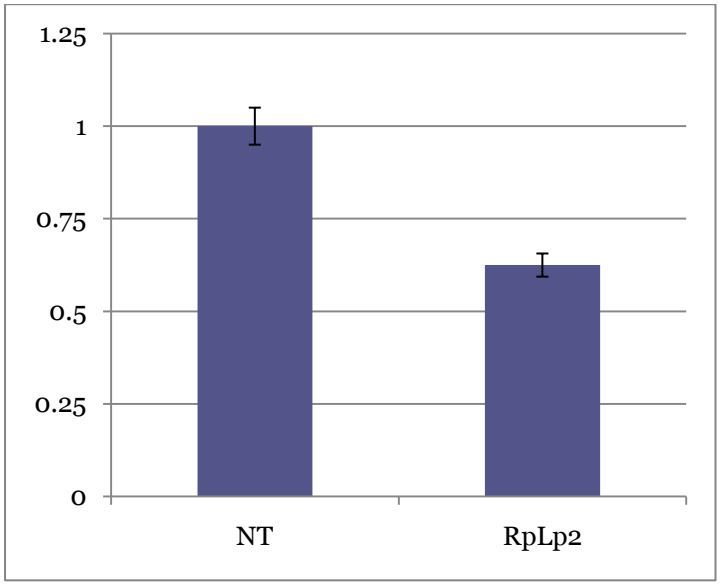
The decrease in Qm mRNA was less significant than in the dm case, moreover no difference was observed between the variant (1) and (2). The RNAi treatment ended up in a decrease of 25% of the constitutive presence of the transcript.



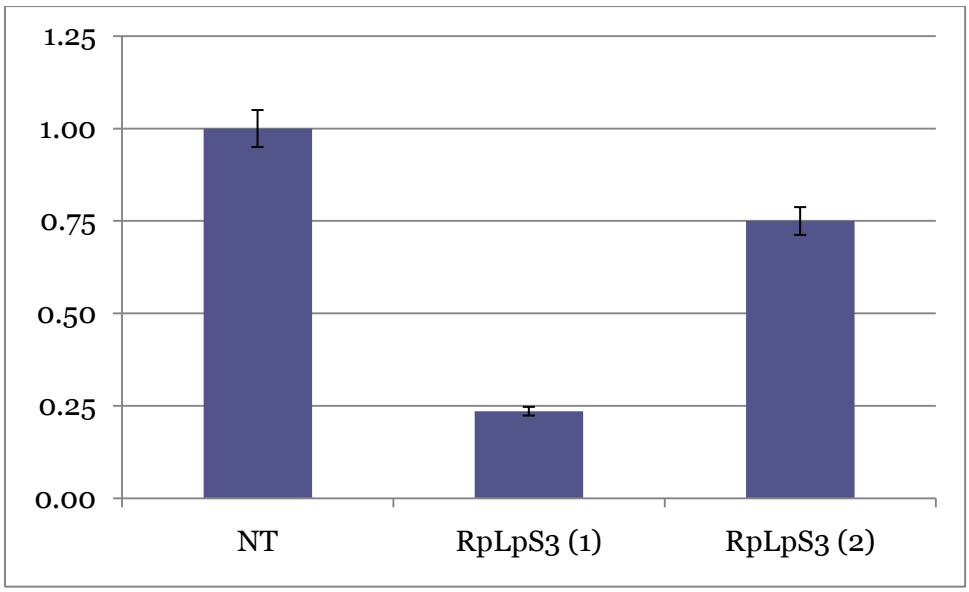
As anticipated in the preliminary observations of the RNAi effects on cell proliferation, the decrease of RpLp0 was quite asymmetric. The construct (1) actually decreased the presence of its endogenous target by ~75% while the variant (2) only by the 30%.



RpLp0 was treated with only one variant of dsRNA, that proved to be mildly effective bringing a reduction of the endogenous transcript of 40%.



Finally, the qPCR on RpLpS3 highlighted a bold difference between variants: as matter of fact, RpLpS3 (2) reduced the presence of its target by 25% whereas RpLpS3 (1) dramatically dropped by 76%.



Subsequent to the analysis of the target presence, cDNA from S2 cells treated for seven days with dm(1) dsRNA was investigated for 5 genes and their putative connection to dm KD.

Further than the 75% decrease in the dm mRNA, it resulted clear how both Nop60 and ppan were drastically diminished by respectively 70 and 50% (Fig 12).

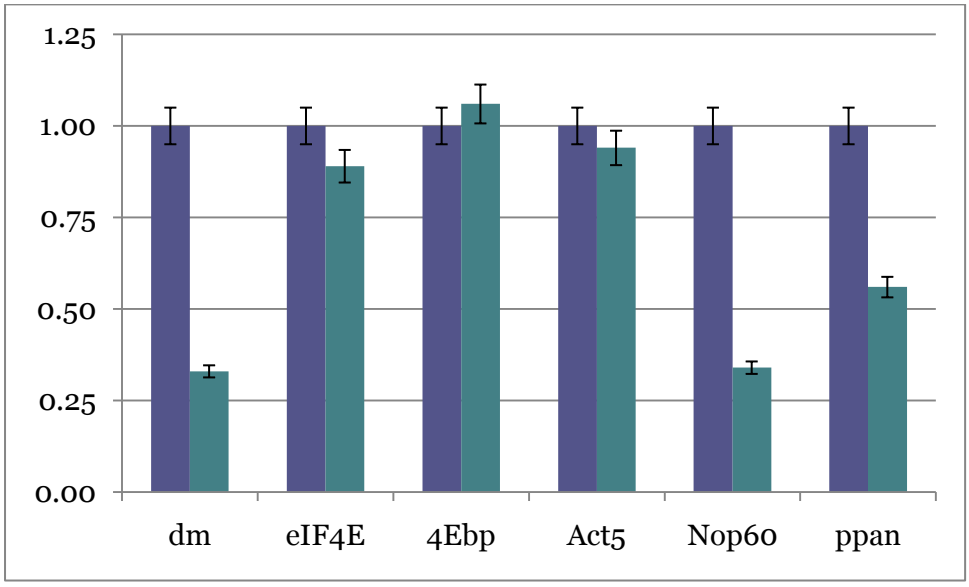


Figure 14: qPCR of related genes

On the contrary, three genes ended up being not affected at all: it is the case of eIF4E, 4Ebp and Act5.

qPCR - flies

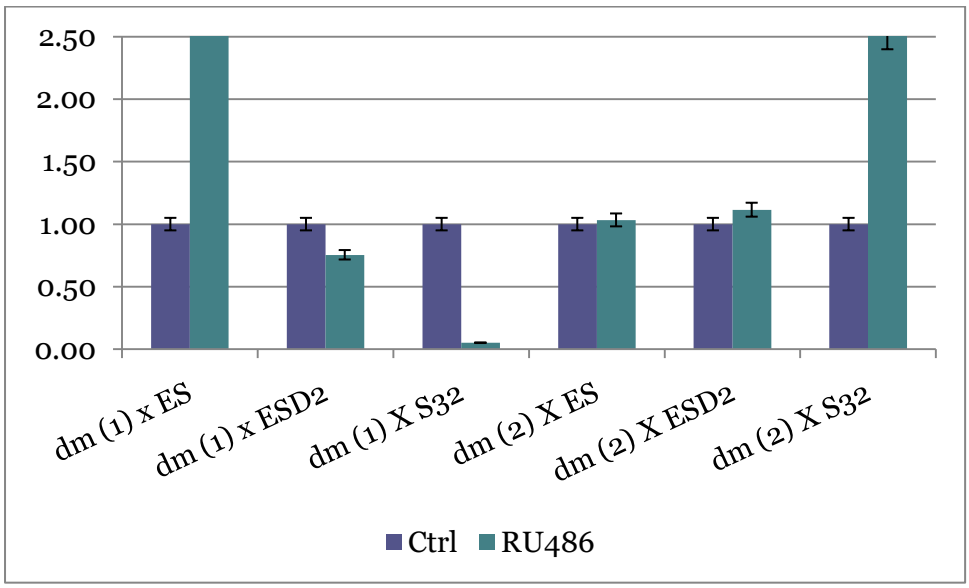
To test the effect of reduced *Myc* expression on lifespan Professor Stephen Helfand (Helfand Lab, Brown University) has obtained two male *Myc* (dm (1) and dm (2)) RNAi stocks developed from the Transgenic RNAi Project (TRiP) at Harvard University, specifically stocks BL25783 and BL25784. The Helfand lab then proceeded mating the two TRiP stocks with females from a panel of stocks containing respectively the following GeneSwitch GAL4 drivers: tubulin (a ubiquitous driver, TB), Elav (a pan-neuronal driver, ES), S32 (a head fat body driver), and S106 (an abdominal fat body driver). This generated 8 stocks in which it is possible to conditionally knock-down *Myc* (dm) expression in adult flies by adding RU-486 to their food. The fruit flies were treated with RU-486 for 4 to 6 days and finally sent me for mRNA extraction, reverse transcription and qPCR, as described previously.

With the aim of validate the most efficient driver for dm silencing several qPCR were done. The cDNA were made out from direct mRNA extraction from either 30 heads or bodies, starting from males.

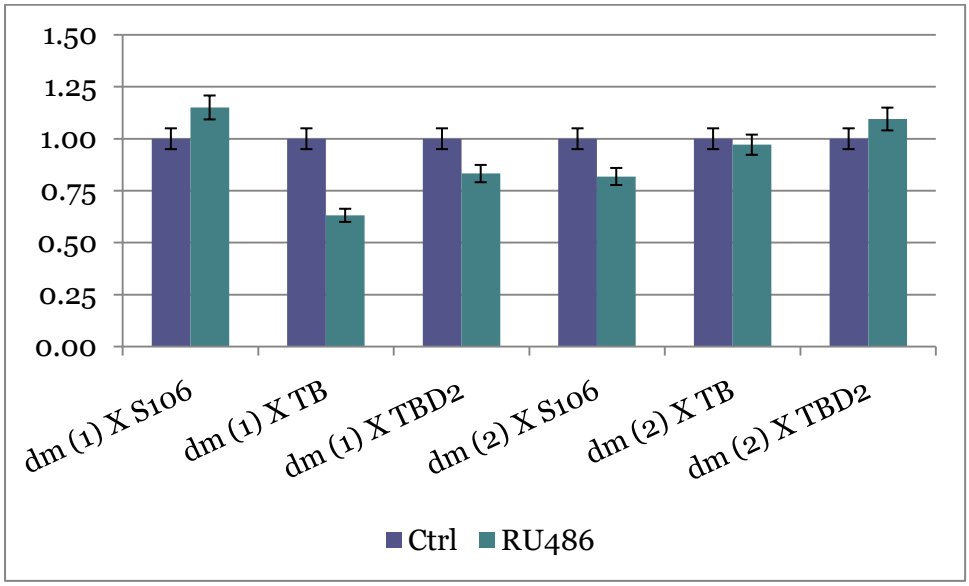
Cross	Tissue	Conc. mRNA (ng/µl)
dm (1) x ES (c)	Heads	6.2
dm (1) x ES (RU)	Heads	6.6
dm (1) x ESD2 (c)	Heads	8.0
dm (1) x ESD2 (RU)	Heads	11.6
dm (1) x S32 (c)	Heads	6.8
dm (1) x S32 (RU)	Heads	7.2
dm (2) x ES (c)	Heads	9.9
dm (2) x ES (RU)	Heads	8.2
dm (2) x ESD2 (c)	Heads	6.2
dm (2) x ESD2 (RU)	Heads	9.0
dm (2) x S32 (c)	Heads	10.7
dm (2) x S32 (RU)	Heads	8.4

Cross	Tissue	Conc. mRNA (ng/µl)
dm (1) x TB (c)	Bodies	45.9
dm (1) x TB (RU)	Bodies	48.2
dm (1) x TBD2 (c)	Bodies	36.4
dm (1) x TBD2 (RU)	Bodies	37.1
dm (1) x S106 (c)	Bodies	36.7
dm (1) x S106(RU)	Bodies	47.1
dm (2) x TB (c)	Bodies	36.0
dm (2) x TB (RU)	Bodies	34.3
dm (2) x TBD2 (c)	Bodies	50.4
dm (2) x TBD2 (RU)	Bodies	39.1
dm (2) x S106 (c)	Bodies	43.9
dm (2) x S106 (RU)	Bodies	40.4

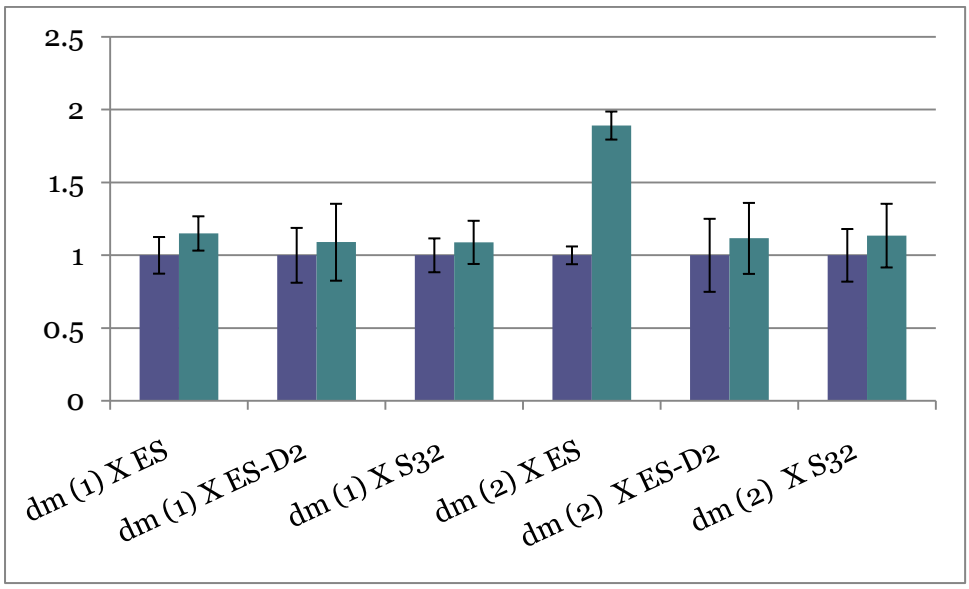
The qPCR reactions on the head identified a driver that decreased by 95% the expression of dm (myc) in the fruit flies head: it is the case of dm (1) crossed with S32. Interestingly, the same driver with a different variant as of dm (2) had the very opposite effect, enhancing the expression of the gene by almost 250%.

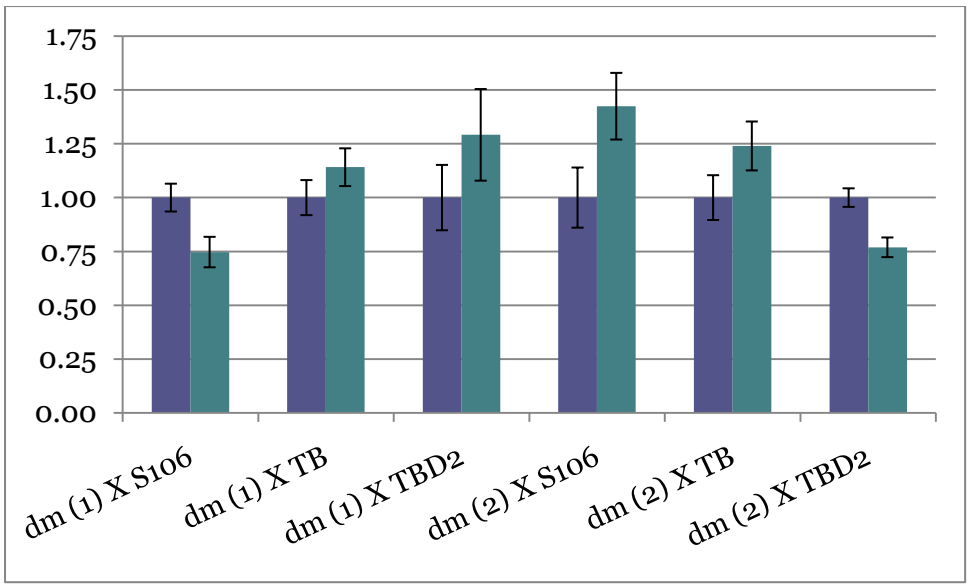


Next, the qPCR didn't show any highly effective driver throughout the whole male body. On the other hand, dm (1) crossed with a Tubulin driver decreased the endogenous presence of the dm transcript by 40%.



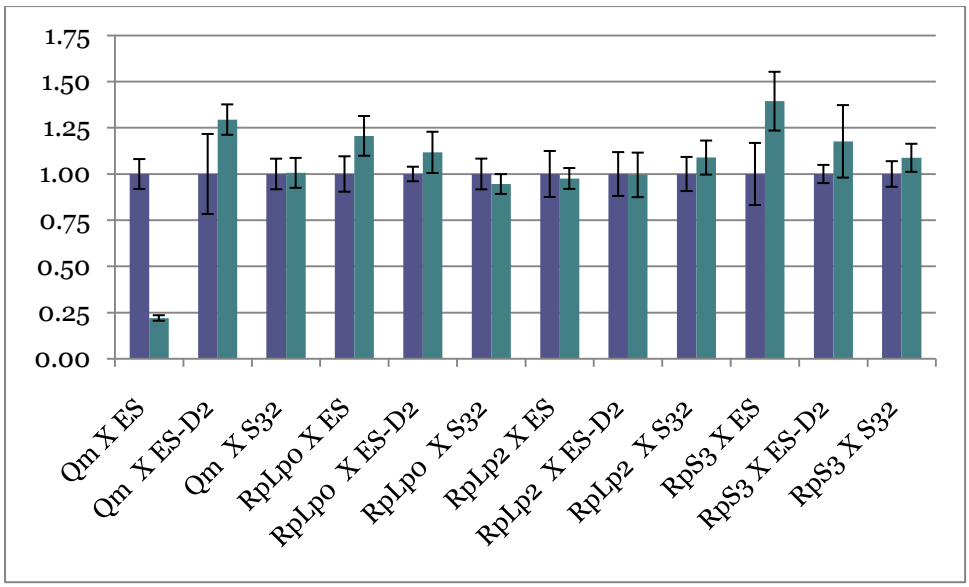
After the identification of an optimal driver of dm silencing in male fruit flies the experiment has been repeated with females. Interestingly no candidate drivers were found in the heads and neither in the whole bodies.

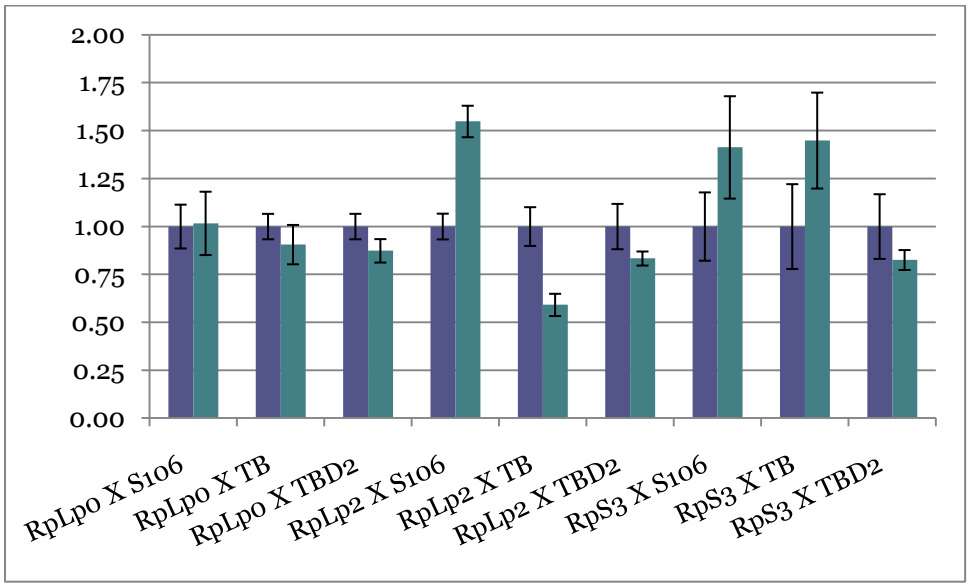




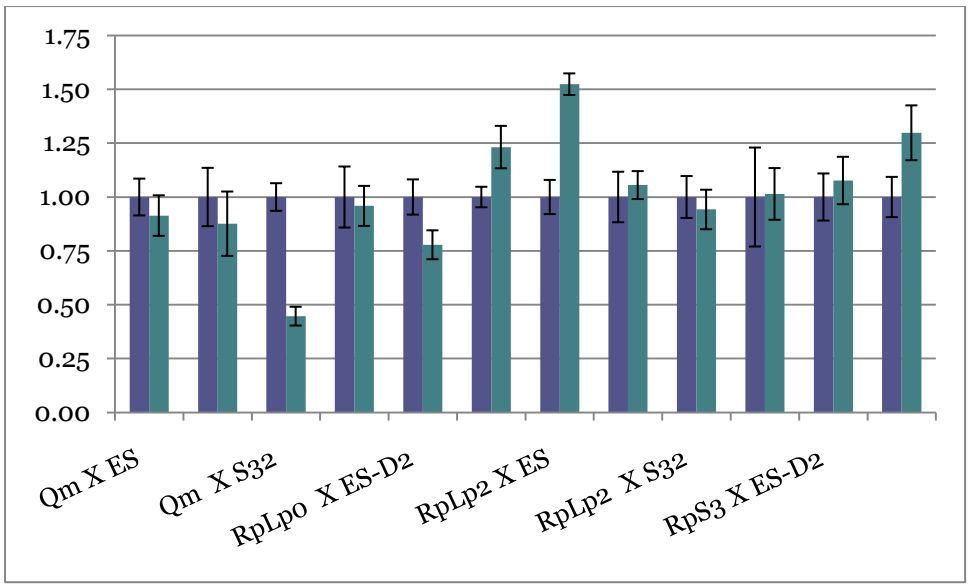
As next step, the Helfand lab bred some more stocks from TRiP with the same drivers. The new stocks tagged along the very same genes used in the cell culture: Qm (BL29356), RpLp0 (JF01335), RpLp2 (JF01516) and RpS3 (JF01410). Males and females were both treated with RU486 and then processed as in the previous experiment. Only in the case of Qm it wasn't possible to test both heads and bodies, but just the heads because of unexpected loss of all the fruit flies carrying that genotype.

No efficient silencing was detected either in male heads or bodies, with isolate exceptions of Qm with the Elav driver in the heads and partially RpLp2 crossed with Tubulin driver.





The analysis run on the females, on the other hand, showed no particularly interesting results in the heads, where just Qm crossed with S32 had decrease of at least 50% of the endogenous transcript. The bodies had a better response to silencing in general but only few highly efficient settings were found: it is the case of RpLp0 and RpS3 crossed with Tubulin D2 driver; in these two cases , the level of target mRNA decreased of almost 50%.


1.75
1.50
1.25
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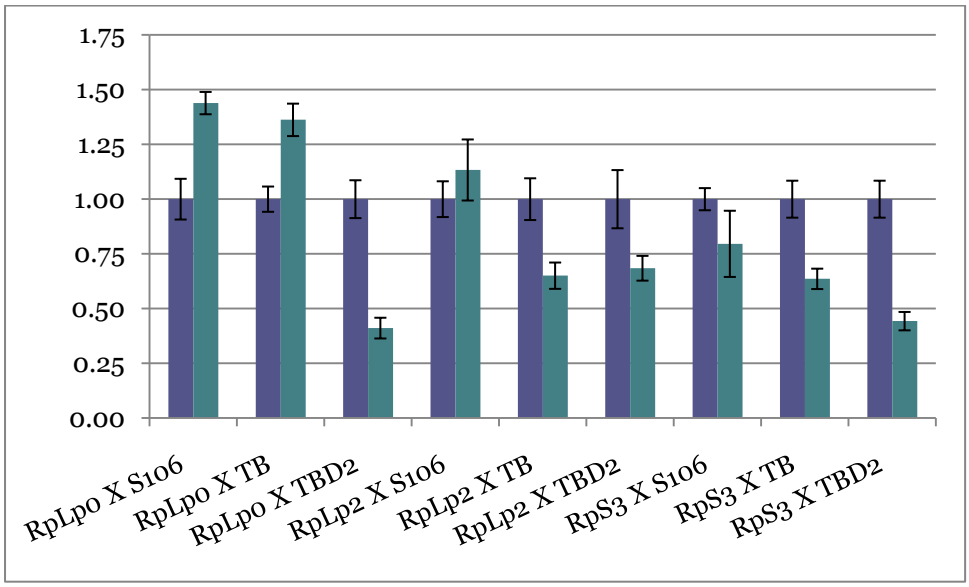
0.50
0.25
0.00
Qm X ES
Qm X S32
RpLp0 X ES-D2
RpLp2 X ES
RpLp2 X S32
RpS3 X ES-D2


1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
RpLp0 X S106
RpLp0 X TB
RpLp0 X TBD2
RpLp2 X S106
RpLp2 X TB
RpLp2 X TBD2
RpS3 X S106
RpS3 X TB
RpS3 X TBD2

Ribosomal RNA quantification

Total RNA from S2 cells treated was loaded into a RNA nano chip, in a Bioanalyzer 2100 (Agilent). The quality score, represented as RNA Integrity Number (RIN) (Schroeder et al., 2006), (Imbeaud et al., 2005) had to be equal or higher than nine to allow the sample to be further analyzed (Fig. 6).

Sample Name	Sample Comment	Status	Result Label	Result Color
WT 10-1		✔	RIN:10	
MYC 10-1		✔	RIN: 9.60	

Figure 15: RIN

A total of 5 samples for group, each with its own technical replicate, were considered for the analysis of the ribosomal RNA content. Moreover each sample was evaluated in its gel-like view in order to become aware of eventual anomalies (Fig. 7).

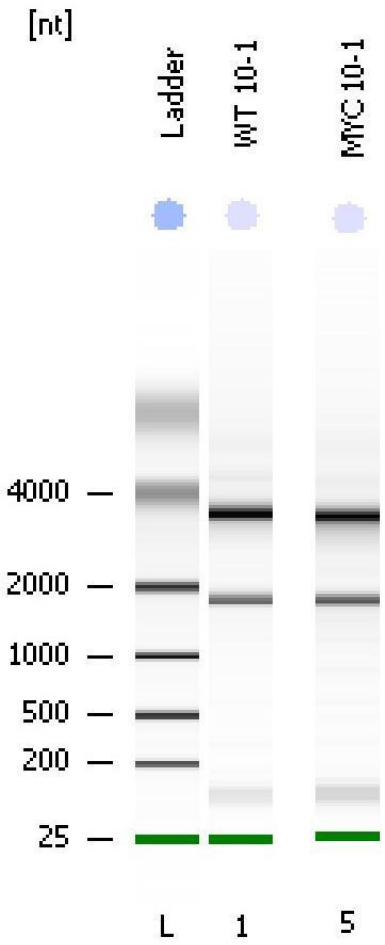


Figure 16: gel-like view of RNA

Then the electropherograms data were exported as raw data and integration of the peaks of ribosomal RNA scored (Fig. 15-16).

A statistically significant difference was found in the total content of ribosomal RNA between the Not Treated S2 cells and the dm (1) treated. The RNAi interference on dm has a negative effect on the cellular ribogenesis for an overall result of a 15% less rRNA compared to the wild type (70% versus 85%, p value < 0.01)

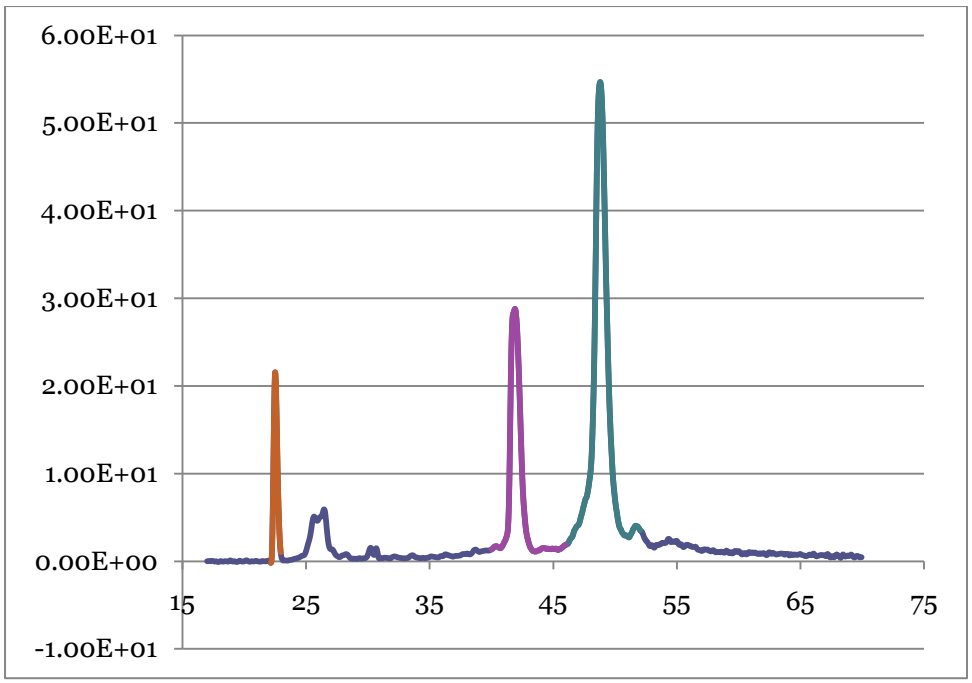


Figure 17: representative NT electropherograms

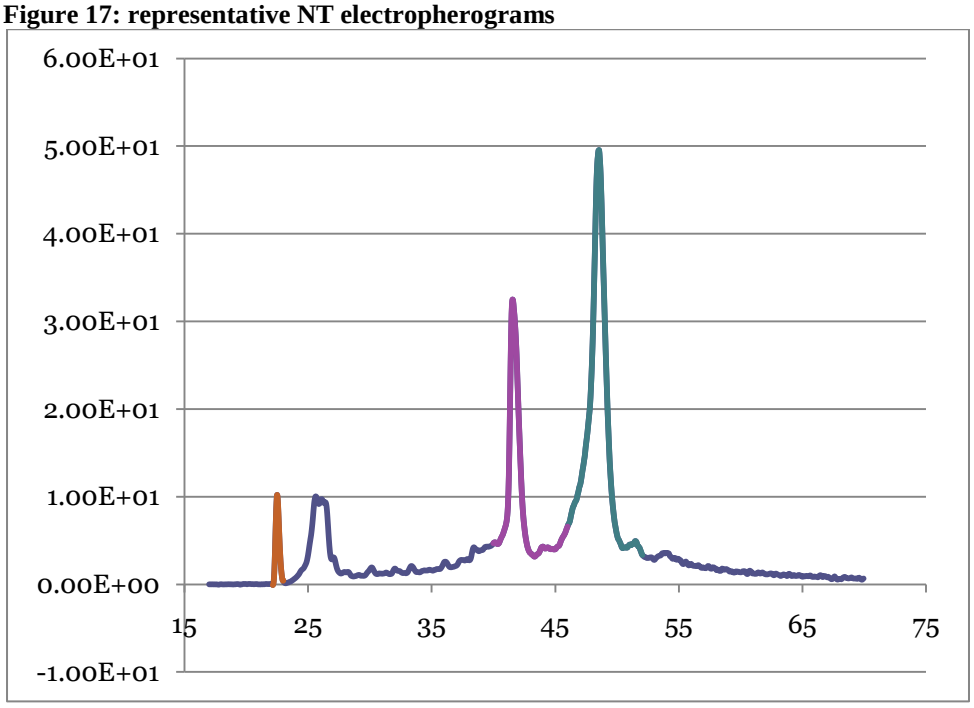


Figure 18: representative dm-treated electropherograms

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Polysome Gradient

First step was to get confident enough with the technique to have results comparable to the ones usually described in literature. To do this several experiment were carried over with Not Treated S2 cells, until the polysome gradient profile recorded at the fractionator was good enough to be used for quantitative comparisons.

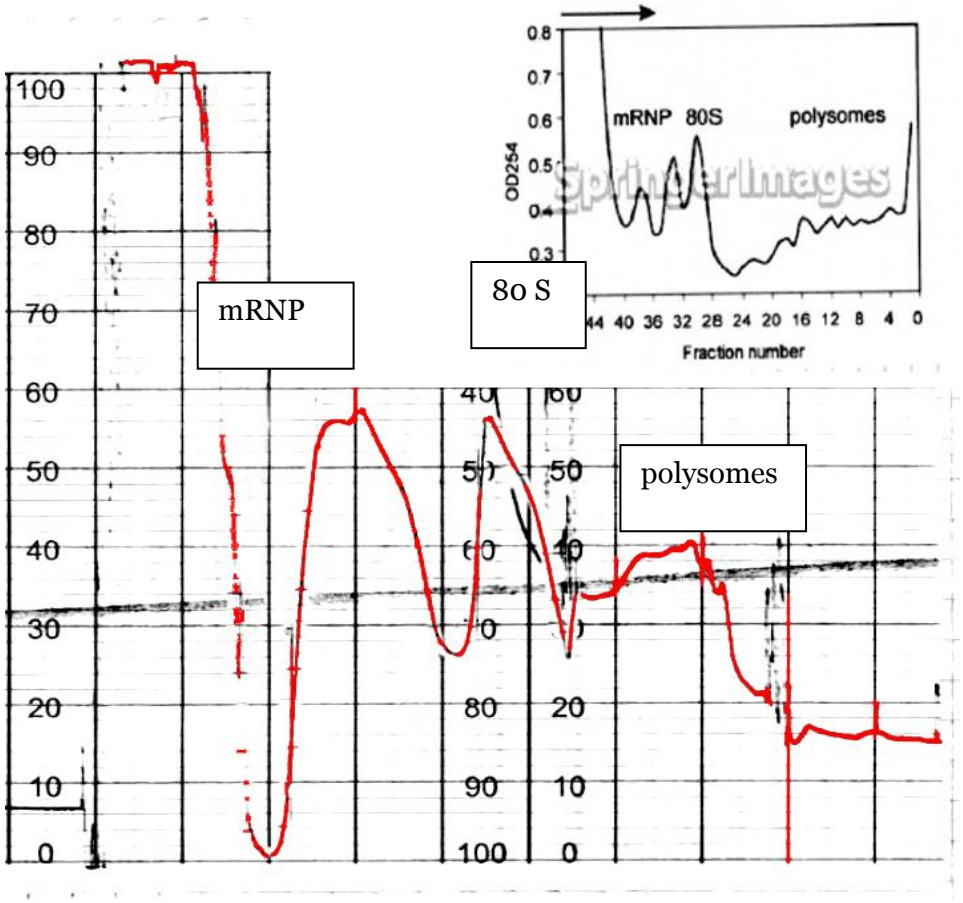


Figure 19: representative gradient profile, in the small box: gradient profile according to literature (courtesy of Springer Images)

Finally, when the technique was reproducible enough, the final experiment consisted in the comparison between the profile of Not Treated S2 cells with dm(1) – treated cells. Each sample had both a technical replica and biological replica.

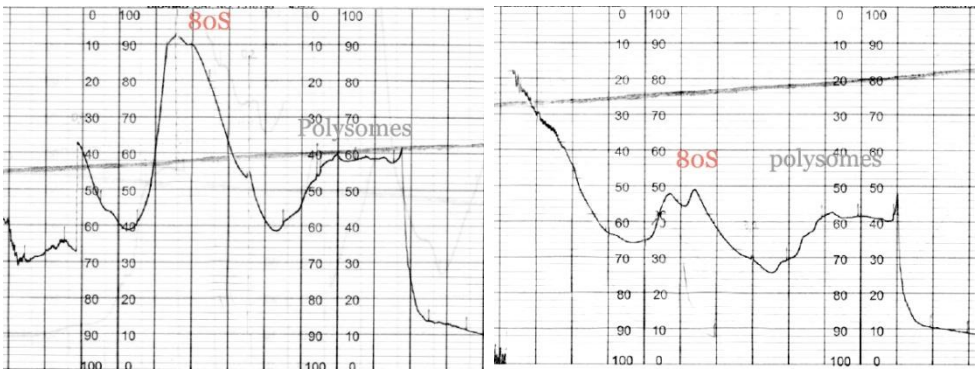


Figure 20: Figure 7: representative gradient profile from Not Treated S2 cells (on the left) and from dm-treated S2 cells (on the right)

It resulted evident how the silencing of dm reduced remarkably the total of available ribosomes, represented by the area underlying the graph. At the same time it shows how despite the diminished units, the dm – treated cells cope with the stress using a much larger fraction of free ribosomes, as shown by the ratio between the polysomes and the 80S areas.

Discussion

The aim of this study was to test whether it was possible to develop an efficient model of study of dm (myc) and its role in the DR longevity pathway (Grandison et al., 2009). It is well known that TOR regulates protein synthesis (Evans et al., 2010), (Stanfel et al., 2009), (Wullschleger et al., 2006), both by controlling the expression of ribosome biogenesis (RiBi) via myc, and by regulating translation initiation via genes such as S6k and 4E-BP.

Myc is also known to regulate both RiBi genes and translation initiation factors (TIFs) such as eIF4E, eIF2α, eIF4AI and eIF4GI. Additionally myc controls transcription of many other genes involved in functions such as cell division, differentiation and cell death, which could in principle have a role in determining lifespan. In the recent years Myc's central role in the regulation of protein synthesis has emerged (van Riggelen et al., 2010). Myc has been shown to regulate protein synthesis at multiple levels by controlling the transcription of ribosomal RNA and proteins, gene involved in processing of ribosomal RNA, genes involved in the nuclear export of ribosomal subunits, and translation initiation factors.

Both the models *in vitro* and *in vivo* proved to be effective in creating the optimal experimental conditions for further analysis in defining the role of each ribosomal protein in the network of interaction with dm (myc).

The production via PCR and T7 RNA polymerase of large quantities of dsRNA proved to be efficient and cost effective. The remarkable expansion in cell diameter in the Rho1 – treated S2 cells was the first positive feedback of the experimental protocol. Additionally we checked for any effect of the dm dsRNA treatment on the cell's phenotype, detecting a small but statistically significant decrease in average cell diameter (5%, p value <0.19). This is consistent with the growth inhibition induced by Myc knock-down (van Riggelen et al., 2010), (Teleman et al., 2008).

An unexpected point of interest is the asymmetrical correlation between cell proliferation and efficiency in gene silencing in different targets: as for RpLpS3, where there is a difference in cytotoxicity and proliferation rate

where RpLpS3 (2) is twice as lethal than the other, and when it comes to silencing the dsRNA variant RpLpS3 (1) proves to be three times more effective than RpLpS3 (2). Quite different is the case of RpLp2 where the transcript RpLp2 (1) has better silencing efficacy but is not linked to a higher cytotoxicity than its counterpart RpLp2 (2). Finally there's the case of dm (myc) where no difference in cell proliferation and mortality corresponds to a very similar level of silencing of the endogenous transcript.

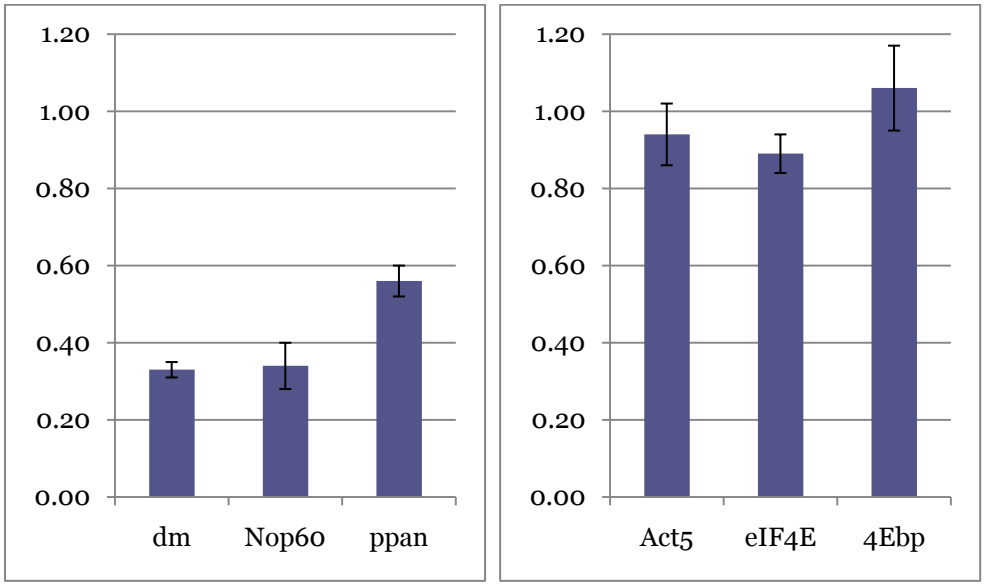
	Mortality rate	Decrease of transcript
RpLpS3 (1)	5.1 %	77.2%
RpLpS3 (2)	9.4 %	25.9%
RpLpo (1)	6.1%	73.5%
RpLpo (2)	5.7%	31.9%
dm (1)	3.8 %	70.6%
dm (2)	2.8 %	75.8%

The robustness of the experimental model *in vitro* has been proved by the qPCR data on specific targets: in dm-treated S2 cells there is a very good agreement between dm (myc) and its target that have been investigated. It is the case of Nop60 and ppan: both are known myc targets in *Drosophila m.* S2 cells and show a statistically significant decrease in their endogenous transcript ($p < 0.01$).

On the other hand, Act5 is known not to be a myc target and the quantitative data on its abundance are evidence for no change at all between dm-treated cells and Not Treated.

Finally the two candidate genes that were investigated for dm-related rearrangements in expression proved no relevant differences: 4Ebp has a slight increase compared to the NT cells (~4%) well above any threshold of significance. Likewise eIF4E has small decrease (~9%) but doesn't pass any t-test and therefore cannot be considered as relevant, as a matter of fact this

result might be of interest because eIF4E, despite its lacking the canonical e-box on promoter region, is known to be a myc target in human but nothing is known about fruit-flies .



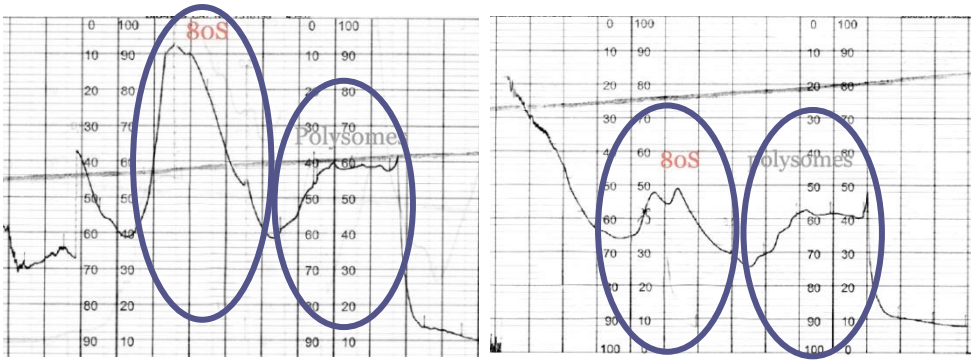
Teleman et al. have demonstrated that a solid link exist between the TOR pathway and Myc. Specifically they showed that in D. *melanogaster* TORC1 appears to regulate Myc primarily at post-transcriptional level, and that *in vivo* Myc acts as a mediator of nutrient-regulated gene expression downstream of TOR. Because the vast majority of these genes are involved in ribosome assembly, they propose a model in which Myc mediates TOR's regulation on biogenesis.

In this context becomes very important the fulfillment of one of the aims in this project, which is the identification of optimal drivers for the silencing of fruit-flies dm *in vivo*. As a matter of fact the Tubulin (ubiquitous) driver from TRiP stocks reduced Myc expression by ~40% after four to six days of treatment with RU486 in male fruit flies. Even better it's the behavior of the S32, a head fat body driver that dramatically dropped Myc expression by 95% with respect to controls. Hence, it is already possible to investigate the effects of Myc knockdown in both adult male and female fruit flies and in at least one tissue (head fat body). In addition, these results open the path for further

research on more tissue specific drivers, and also determine the amount of RU486 for optimal Myc knockdown.

Furthermore we measured the level of ribosomal RNA in both Myc knockdown cells and Not Treated S2 cells via Bioanalyzer. The results showed the typical *Drosophila melanogaster* RNA profile, highlighting the statistically significant difference in ribosome abundance (~15%) between the two conditions, with Myc depletion leading to a much lower number of ribosome at a cellular level.

To better understand how ribosome depletion translated into changes in protein synthesis, it was measured the fractionation profile from 10-45% sucrose gradients from S2 cells dm-treated and controls as described by Bronson et al. (Bronson et al., 2010), sucrose density gradients separated the ribosomes into the subpolysomal / monosome and polysome fractions.



The ratio of polysomes to monosomes was relatively low, as previously reported in literature in normal S2 cells (Patel et al., 2009). The lower levels in the polysome profiles in Myc knockdown cells confirmed the overall ribosomal biogenesis inhibition detected with Bioanalyzer. However, while the overall level of ribosomes decreased, there were relatively more polysomes than monosomes in Myc knockdown than in controls, indicative of an increase of ribosomes' translational efficiency in Myc depleted cells.

This is very interesting and opens the door to different possible interpretation as a compensatory mechanism that tries to counter the reduced availability of ribosomes by boosting translational efficiency of some or all mRNAs.

Conclusion

The relatively large amount of work done on this project has to be considered only the first step since it has been considered as the preliminary result in a 5-year grant submission to the National Institute of Health.

Considering the experimental aims of the research, all collected data allow to draw some interesting conclusions. Both the *in vitro* and *in vivo* approaches showed their flexibility and effectiveness: large production of dsRNA via PCR or crossings among TRiP stocks are relatively easy to handle and optimize. Moreover they allow working on very large amount of subjects in very cost-effective way.

A consistent decrease of 75% of endogenous transcript in cells treated with dm – dsRNA is the optimal environment for further analysis in myc's control of protein synthesis, as our results with polysome gradients clearly show.

Moreover the identification of highly efficient drivers throughout either the whole body, like Tubulin, or in a tissue specific way, like S32 allows to investigate the effect of Myc knockdown in adult flies.

These results are the fundamental basis for further goals of the project, like as identify the Myc targets within the protein pathway that modulate lifespan using a combination of ChIP-seq and RNA-seq. A second crucial goal will be to identify what translational changes occur when lifespan is extended by inhibiting Myc activity, using the knowledge of the previous aim and combining it to measurement of translation rates via 35S method and polysome gradients.

With these considerations, the 5-year grant will allow to gather critical data on Myc's control of protein synthesis via regulation of ribosomal biogenesis and translation initiation factors, and its effect on longevity.

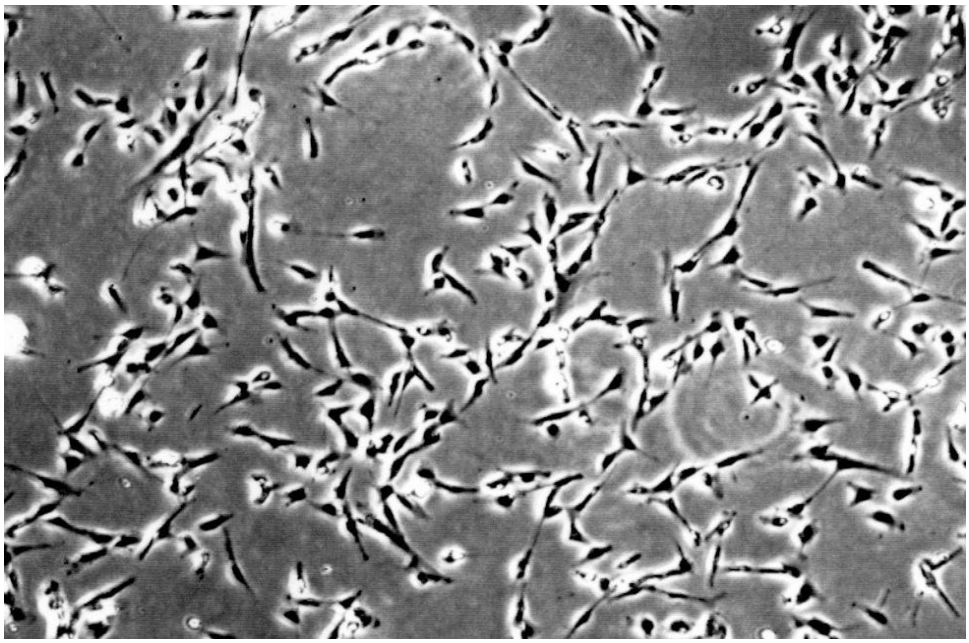
Bibliography

1. Bronson MW, Hillenmeyer S, Park RW, Brodsky AS. Estrogen coordinates translation and transcription, revealing a role for NRSF in human breast cancer cells. Mol Endocrinol. 2010 Jun;24(6):1120-35. Epub 2010 Apr 14.
2. Chen C, Liu Y, Zheng P. mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells. Sci Signal. 2009;2(98):ra75.
3. Dang CV, O'Donnell KA, Zeller KI, Nguyen T, Osthus RC, Li F. The c-Myc target gene network. Semin Cancer Biol. 2006;16(4):253-64.
4. Echeverri, C.J. & Perrimon, N. High-throughput RNAi screening in cultured cells: a user's guide. Nat. Rev. Genet. 7, 373–384 (2006).
5. Elbashir, S.M. et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494–498 (2001).1,2.
6. Estep PW, Warner JB, Bulyk ML. Short-term calorie restriction in male mice feminizes gene expression and alters key regulators of conserved aging regulatory pathways. PLoS One. 2009;4(4):e5242. PMCID: 2667255.
7. Evans DS, Kapahi P, Hsueh WC, Kockel L. TOR signaling never gets old: Aging, longevity and TORC1 activity. Ageing Res Rev. 2010.
8. Fire, A. et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806–811 (1998).
9. Grandison RC, Piper MD, Partridge L. Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. Nature. 2009;462(7276):1061-4. PMCID: 2798000.
10. Grandori C, Cowley SM, James LP, Eisenman RN. The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu Rev Cell Dev Biol. 2000;16:653-99.

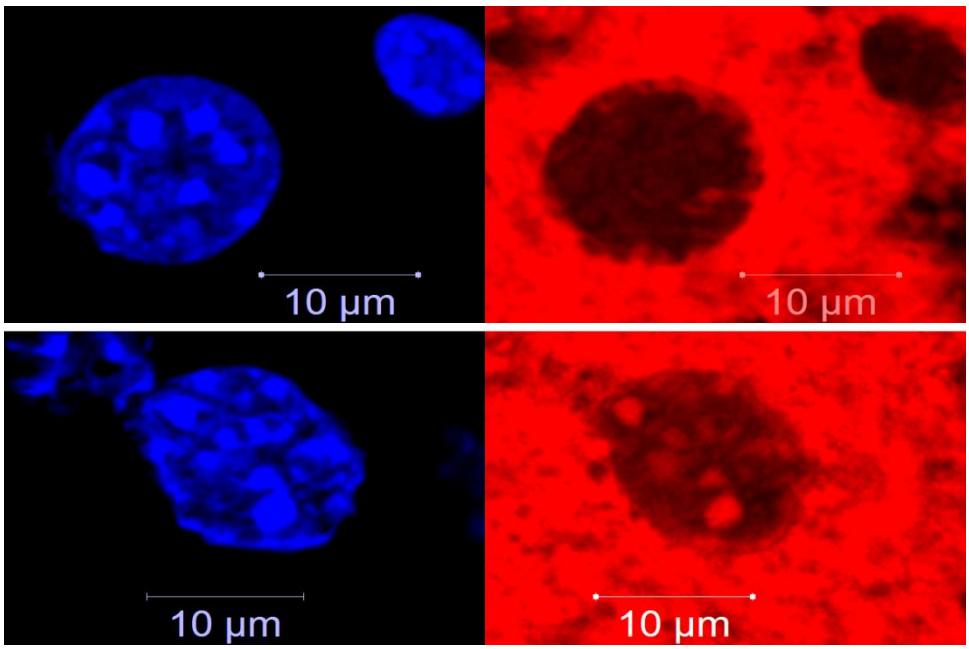
11. Hansen M, Chandra A, Mitic LL, Onken B, Driscoll M, Kenyon C. A role for autophagy in the extension of lifespan by dietary restriction in C. elegans. PLoS Genet. 2008;4(2):e24. PMCID: 2242811.
12. Hansen M, Taubert S, Crawford D, Libina N, Lee SJ, Kenyon C. Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans. Aging Cell. 2007;6(1):95-110.
13. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009;460(7253):392-5. PMCID: 2786175.
14. Horn T, Arziman Z, Berger J, Boutros M. GenomeRNAi: a database for cell-based RNAi phenotypes. http://genomernai.de/GenomeRNAi/
15. Imbeaud S, Graudens E, Boulanger V, Barlet X, Zaborski P, Eveno E, Mueller O, Schroeder A, and Auffray C. Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces, Nucl. Acids Res. 2005 33: e56.
16. Kaeberlein M, Powers RW, 3rd, Steffen KK, Westman EA, Hu D, Dang N, et al. Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. Science. 2005;310(5751):1193-6.
17. Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S. Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Curr Biol. 2004;14(10):885-90. PMCID: 2754830.
18. Lyne R, Smith R, Rutherford K, Wakeling M, Varley A, Guillier F, et al. FlyMine: an integrated database for Drosophila and Anopheles genomics. Genome Biol. 2007;8(7):R129. PMCID: 2323218.
19. Meyer N, Penn LZ. Reflecting on 25 years with MYC. Nat Rev Cancer. 2008;8(12):976-90.

20. Pan KZ, Palter JE, Rogers AN, Olsen A, Chen D, Lithgow GJ, et al. Inhibition of mRNA translation extends lifespan in Caenorhabditis elegans. Aging Cell. 2007;6(1):111-9. PMCID: 2745345.
21. Patel PH, Costa-Mattioli M, Schulze KL, Bellen HJ. The Drosophila deoxyhypusine hydroxylase homologue nero and its target eIF5A are required for cell growth and the regulation of autophagy. J Cell Biol. 2009;185(7):1181-94. PMCID: 2712966.
22. Ramet, M. et al. Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E. coli. Nature 416, 644–648 (2002).
23. Rogers SL and Rogers GC. Culture of Drosophila S2 cells and their use for RNAi-mediated loss-of-function studies and immunofluorescence microscopy. Nat Protoc. 2008;3(4):606-11.
24. Rogers SL, Wiedemann U, Stuurman N, Vale RD. Molecular requirements for actin-based lamella formation in Drosophila S2 cells. J Cell Biol. 2003 Sep 15;162(6):1079-88.
25. Schneider, I. Cell lines derived from late embryonic stages of Drosophila melanogaster. J. Embryol. Exp. Morphol. 27, 353–365 (1972).
26. Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M and Ragg T. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Molecular Biology 2006, 7:3
27. Selman C, Tullet JM, Wieser D, Irvine E, Lingard SJ, Choudhury AI, et al. Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. Science. 2009;326(5949):140-4.
28. Stanfel MN, Shamieh LS, Kaeberlein M, Kennedy BK. The TOR pathway comes of age. Biochim Biophys Acta. 2009;1790(10):1067-74.
29. Steffen KK, MacKay VL, Kerr EO, Tsuchiya M, Hu D, Fox LA, et al. Yeast life span extension by depletion of 60s ribosomal subunits is mediated by Gcn4. Cell. 2008;133(2):292-302. PMCID: 2749658.

30. Storey JD, Tibshirani R. Statistical significance for genomewide studies. Proc Natl Acad Sci U S A. 2003;100(16):9440-5. PMCID: 170937.
31. Teleman AA, Hietakangas V, Sayadian AC, Cohen SM. Nutritional control of protein biosynthetic capacity by insulin via Myc in Drosophila. Cell Metab. 2008;7(1):21-32.
32. van Riggelen J, Yetil A, Felsher DW. MYC as a regulator of ribosome biogenesis and protein synthesis. Nat Rev Cancer. 2010;10(4):301-9.
33. Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L, Muller F. Genetics: influence of TOR kinase on lifespan in C. elegans. Nature. 2003;426(6967):620.
34. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell. 2006;124(3):471-84.
35. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell. 2006;124(3):471-84.
36. Zid BM, Rogers AN, Katewa SD, Vargas MA, Kolipinski MC, Lu TAl. 4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in Drosophila. Cell. 2009;139(1):149-60. PMCID: 2759400.



Homo sapiens & Mus musculus



Part 3 – Development of a biomarker for senescence

Introduction: why stain proteins

In time normal diploid cell lines as fibroblasts have been studied extensively. It is well known that normal somatic cells, with the exception of germ cells and some stem cells, display a finite replicative capacity. This phenomenon was first described in cultured human fibroblasts (Hayflick and Moorhead, 1961), and is commonly referred to as replicative cellular senescence. Their differentiation sequence from mitotic to postmitotic fibroblasts was documented in chicken, mice, rats and man (Bayreuther et al., 1991), and thus were always considered as optimal representative models for studies of general or special issues of cell biology, such as aging. Cellular senescence has been studied predominantly in cell culture (in vitro) under a variety of pathological and normal conditions (Sedivy et al., 2008), (Jun et al., 2011) (Nardella et al., 2011). Among other features, early studies revealed that aging of the cells corresponded to remarkable changes in size and shape (Hayflick, 1968), (Hayflick, 1972), (Cristofalo and Pignolo, 1993) involving an adjustment in cellular protein or at least an alteration of their balance inside the aging fibroblasts.

Early studies aimed to measure first any changes in rates of both synthesis and turnover between late and early passage cells, finding that the senescent cells were consistently more altered thus supporting those theories of cellular senescence which predict altered protein metabolism as a major consequence of the aging process (Dell'orco et al., 1976). Deepening the analysis it turned out how not only the rate of protein synthesis is reduced in senescent cultures, but the average rate of protein degradation is also considerably slowed down: the protein content of senescent cells increases by 1.8-fold as compared to young cells, while the average cell volume is increased even more (almost 5-fold). (Kraftory et al., 1978). Biochemical analysis conducted in our lab confirmed the increase in protein content in whole cells and compared the nuclear fractions of early passage and senescent cells, proving an increase of ~3-folds.

Interestingly enough to our knowledge no one else has ever investigated the protein content on a single cell level: an important limiting factor has been the lack of a well developed and accepted staining method for total protein suitable for fluorescence microscopy. This is in contrast to DNA, where dyes based either on intercalation (acridine) or minor groove binding (DAPI, Hoechst) have been widely used for some time, and have enabled many high resolution as well as quantitative studies in numerous applications. While a number of sensitive staining methods have been developed for protein quantification in solution as well as on solid supports (Noble et al., 2009), these principles have not been extended and generalized for fluorescence microscopy. Therefore we decided to develop a flexible assay meant to easily assess the quantity of protein per cell, and in particular, inside the nucleus. After the screening of several fluorescent dyes with different chemistry of interactions with proteins like Fluorescamine, OPA and QCBCA, our attention was driven onto NanoOrange® (Invitrogen).

It is a merocyanine dye that produces a large increase in fluorescence quantum yield upon interaction with detergent-coated proteins (Jones et al., 2003). It stood up among others because of its wide range sensibility, its insensitivity to nucleic acid and, above all, for its ease of use. The aim of this work was to develop an assay that allows to measure single cell protein content via immunofluorescence. Besides we considered of extreme interest the possibility to couple the newly developed assay with a classical immunofluorescence experiment so as to have information about specific targets presence and relate them to a global condition of the cell.

By doing this we showed how the extent of the total protein staining is maintained whilst we investigated well recognized markers as macroH2A, Lamin A/C and histone H3 in early passage and senescent cells.

Furthermore we conducted the whole work on two different cell lines of fibroblasts as human lung and mouse tail in order to draw attention to the flexibility and the potential of the assay here presented.

The point of the project was to develop a reliable assay: to this extend several attempts were made with different approaches, methods and dyes. At the

same way, not all the experiments and results are reported in the final paper: so a brief recap of the rest of the work done is summed in the Supplemental Data part.

Appendix 1 – Preliminary data

Defining the protocol

It took some time to define the best dye in order to stain cellular proteins. This is mainly due to the large availability of different products on the market; as a matter of fact they rely on different chemistries and various chromophores. Moreover no commercial dye has been designed to fulfill exactly our experimental aims: indeed they are meant to quantify protein in solution or in a gel, while our purpose was to quantify cells in situ on a single cell scale.

During the experimental activity we started from dyes that react with primary amine groups on proteins, such as Fluorescamine, OPA (o-phthaldialdehyde) and CBQCA. Only afterwards we changed principle, using NanoOrange that binds to detergent coating on proteins and hydrophobic regions of proteins.

Most of the preliminary work isn't mentioned in the article journal published at the end of the project that's the reason why it will be written here in form of Supplemental Information.

Fluorescamine

Fluorescamine is intrinsically nonfluorescent but reacts rapidly with primary aliphatic amines to yield a blue-green-fluorescent derivative with excitation/emission maxima ~380/464 nm.

Molecular Formula:	**$C_{17}H_{10}O_4$**
Molecular Weight:	278.2636
CAS Name:	Spiro(furan-2(3H),1'(3'H)-isobenzofuran)-3,3'-dione, 4-phenyl

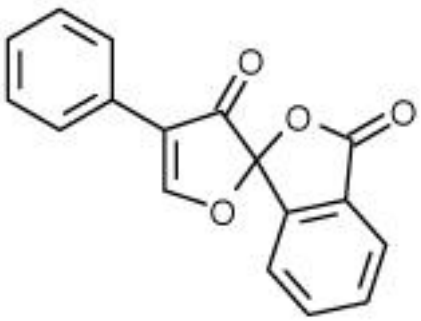


Figure 21: Fluorescamine molecular structure

Despite being widely used in the past (Kagan et al., 2002),(Cesur et al., 2002), fluorescamine has several issues like as the pH of the buffer: when pH increases, excess fluorescamine is more rapidly hydrolyzed, forming a product that is unreactive toward amines. At low pH, protonation of amines further interferes with the fluorescamine derivatization reaction.

All in all, the reagent is quite unstable and it isn't compatible with amine-containing buffers (like Tris or glycine).

Various protocols have been tested but no reproducibility nor stability has been detected. Cells are stained unevenly both in early passage (passage 15) human Lung Fibroblast cells (hLF1) and senescent cells (passage 52). Therefore the dye was rejected and replaced.

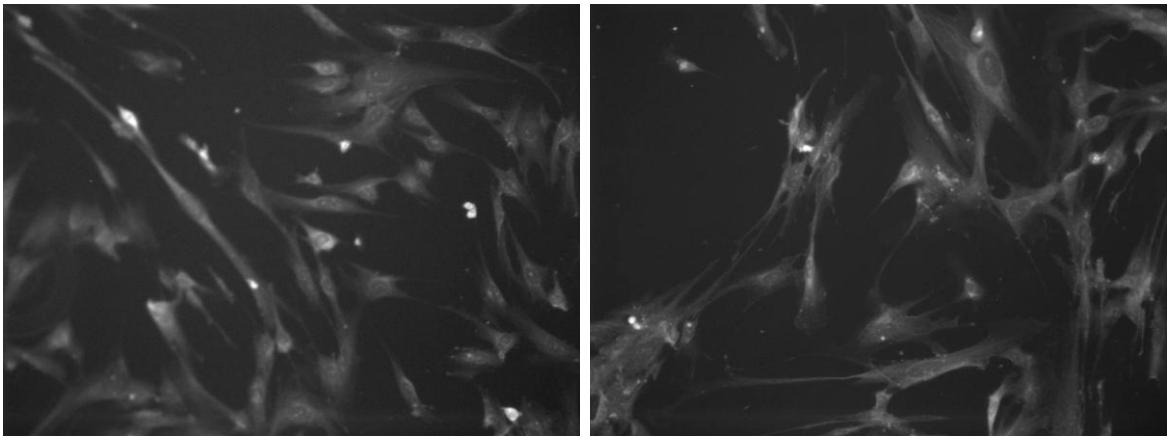


Figure 22: representative image of Early Passage LF1 stained with fluorescamine

OPA (o-phthaldialdehyde)

The aromatic dialdehyde o-phthaldialdehyde (OPA) is essentially nonfluorescent until reacted with a primary amine in the presence of excess cyanide or a thiol, to yield a fluorescent isoindole with excitation/emission maxima ~334/455 nm.

Molecular Formula:	**$C_8H_6O_2$**
Molecular Weight:	134.1342
CAS Name:	1,2-Benzenedicarboxaldehyde

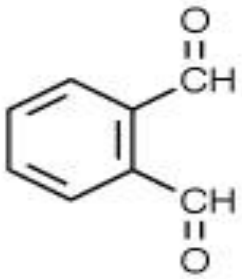


Figure 23: OPA molecular structure

OPA is still used very often (Go et al., 2008), (Yamamoto et al., 2008), (Hapuarachchi and Aspinwall, 2007), because of its fast kinetic properties, its ease of use and low cost. Although despite all these advantageous features, OPA resulted not suitable for our purposes since the signal was not stable and reproducible.

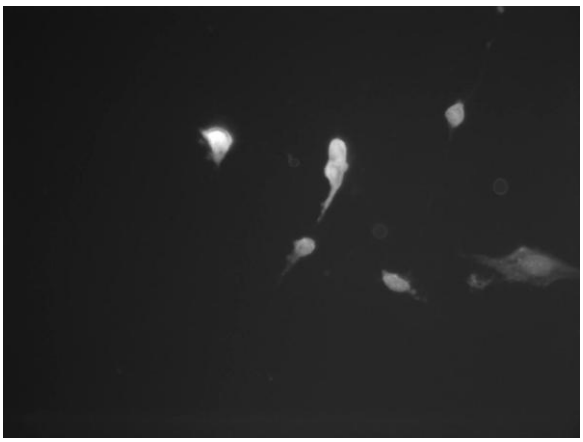


Figure 24: representative image of Early Passage LF1 stained with OPA

CBQCA Protein Quantitation Kit

The CBQCA Protein Quantitation Kit (Molecular Probes ®, cat. No. C-6667) is a very sensitive assay for quantitating proteins in solution, capable of detection as low as 10 ng of protein per mL (Graves et al., 2005), (Huang et al., 2006). Similar in sensitivity to another dye, NanoOrange, CBQCA is supposed to be better suited for accurate quantitation of proteins in the presence of lipids and for lipoproteins.

Molecular Formula:	**$C_{18}H_{11}NO_4$**
Molecular Weight:	305.2892
CAS Name:	N / A

Regardless of its many features, as sensitivity and linearity, the Atto-Tag ™ CBQCA derivatization reagent failed in meeting the experimental goals. No statistical analysis could be done on single cell level so the dye was replaced.

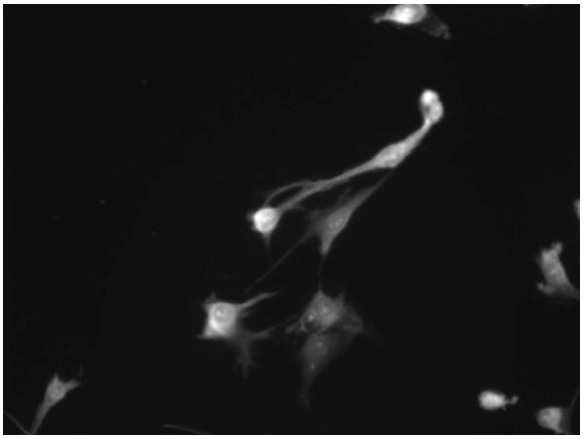


Figure 25: representative image of Early Passage LF1 stained with Atto-Tag™ CBQCA

According to our results, the reason for the failure of all the previous dyes relies on the staining reaction itself: all of them are meant to work at room temperature in a "pure" enviroment, like as protein in solution. For our purposes we had to adjust this since the staining of protein in situ requires a different level of flexibility.

Bearing these consideration we changed approach and moved to a dye that binds to detergent coating on proteins and hydrophobic regions of proteins; moreover the staining step requires a 95°C denaturation step, differently from all the other tested methods.

NanoOrange protein quantitation assay

The NanoOrange Protein Quantitation Kit (Molecular Probes ®, cat. No. N-6666) contains a very sensitive and easy assay for protein quantitation, with detection as low as 10 ng/mL of protein in solution. During the preliminary experiments it resulted perfect for our purposes, allowing a neat and constant detection of both cytoplasmic and nuclear proteins.

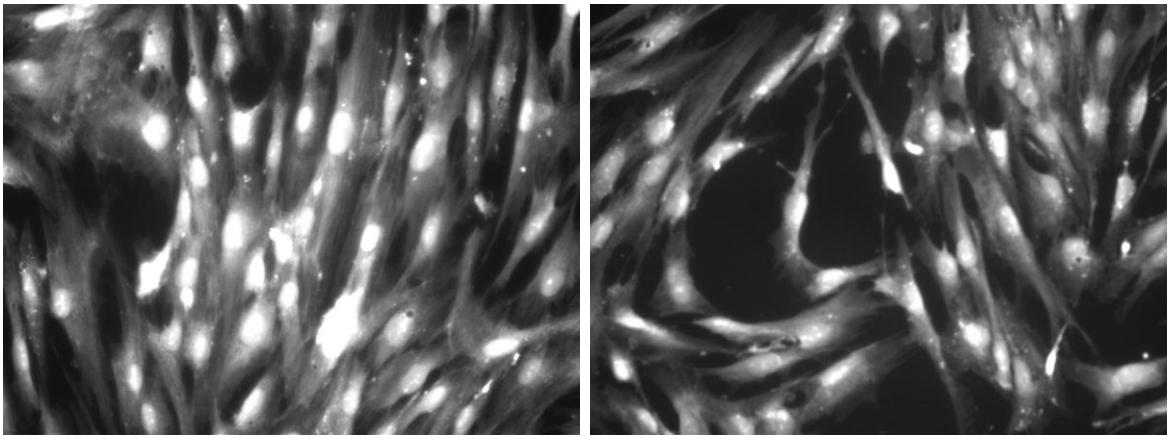


Figure 26: representative image of Early Passage LF1 stained with NanoOrange®

The detergent mixture used as diluent in the staining step as for the dye itself (a merocyanine) are both covered by patent. From the very beginning the assay looked promising; the analysis was then performed on a confocal microscope rather than a fluorescence one, so to improve image resolution and reduce cross-talking between dyes. The protocol required some fine tuning to identify the optimal working condition, all the steps are described in the final paper in the Supplemental Data.

Once the optimal protocol was defined several hundreds of images were collected and analyzed, both for the normal human diploid fibroblast mentioned above (LF1) and primary adult mouse tail fibroblasts (MTF).

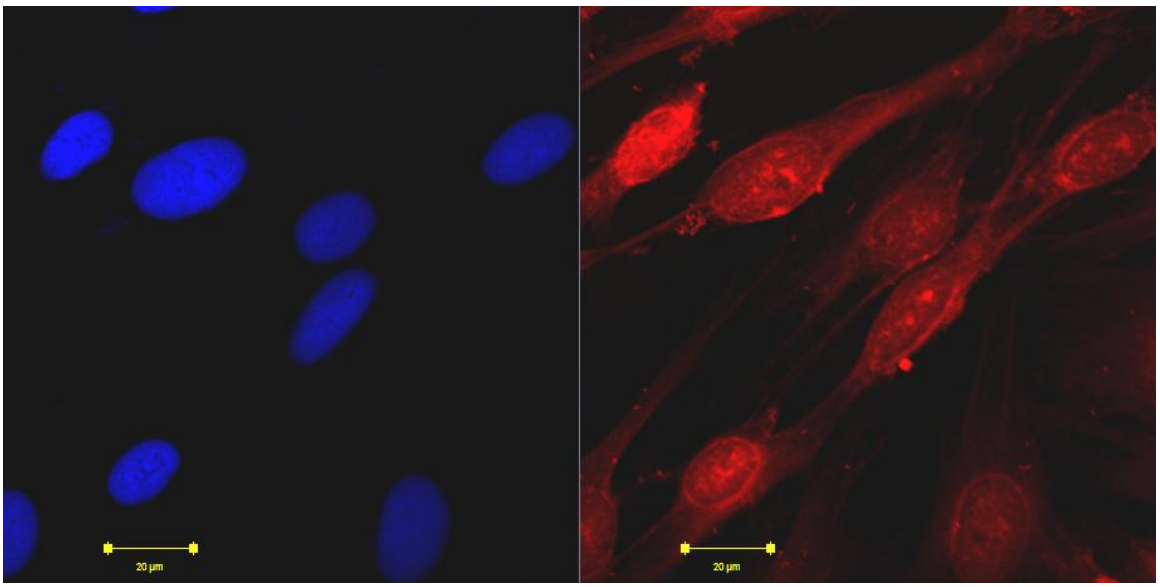


Figure 27: representative image of Early Passage LF1. On the left, DAPI counter stains the DNA. On the right, NanoOrange stains cellular proteins. [bar = 20µm]

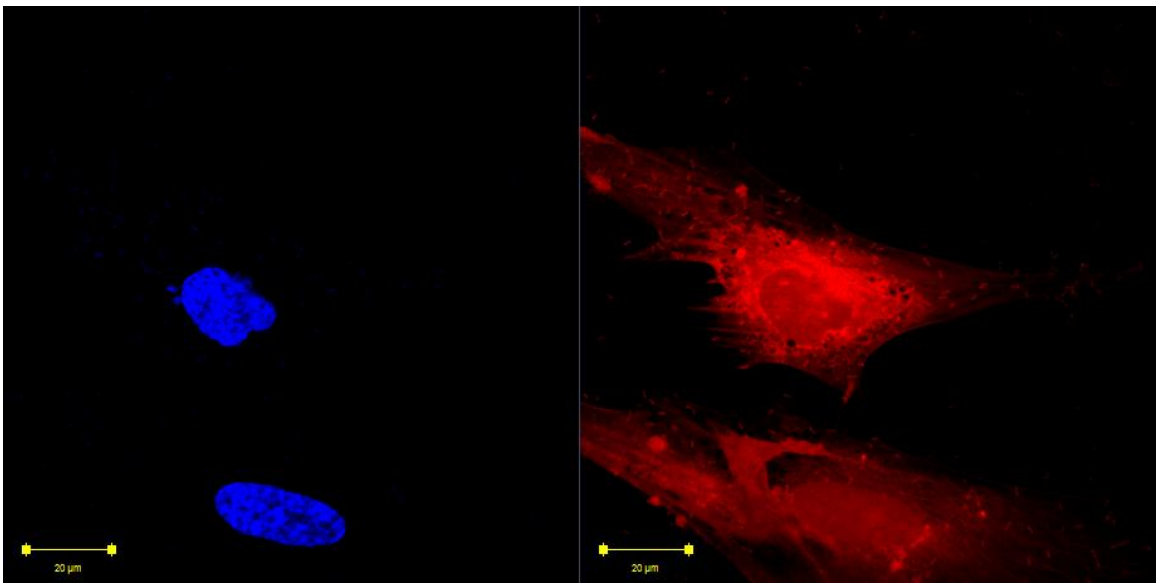


Figure 28: representative image of Senescent LF1. On the left, DAPI counter stains the DNA. On the right, NanoOrange stains cellular proteins. [bar = 20µm]

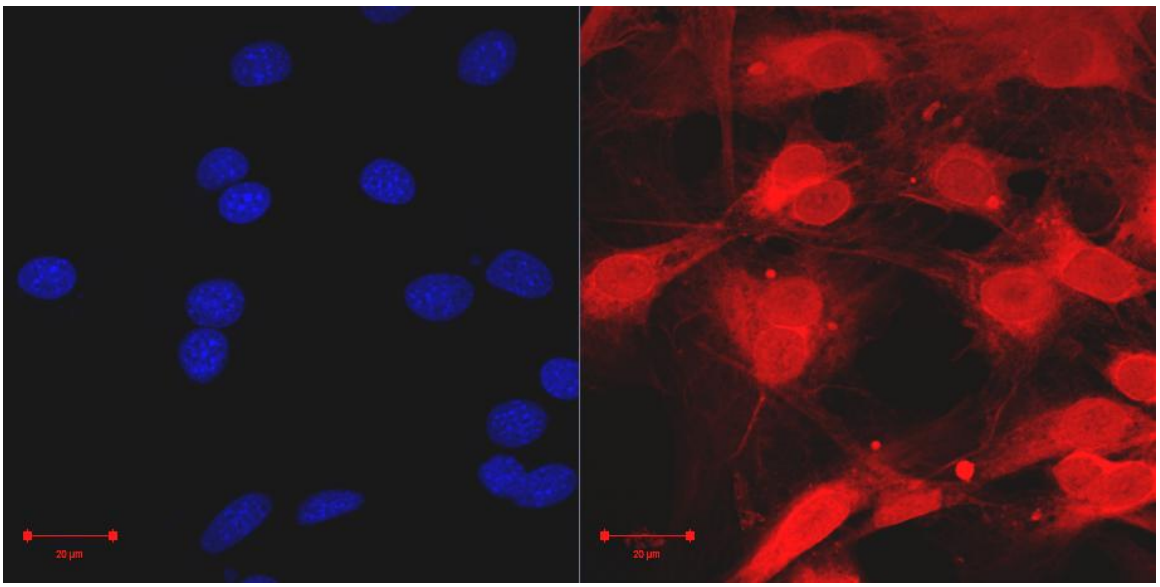


Figure 29: representative image of Early Passage MTF. On the left, DAPI counter stains the DNA. On the right, NanoOrange stains cellular proteins. [bar = 20µm]

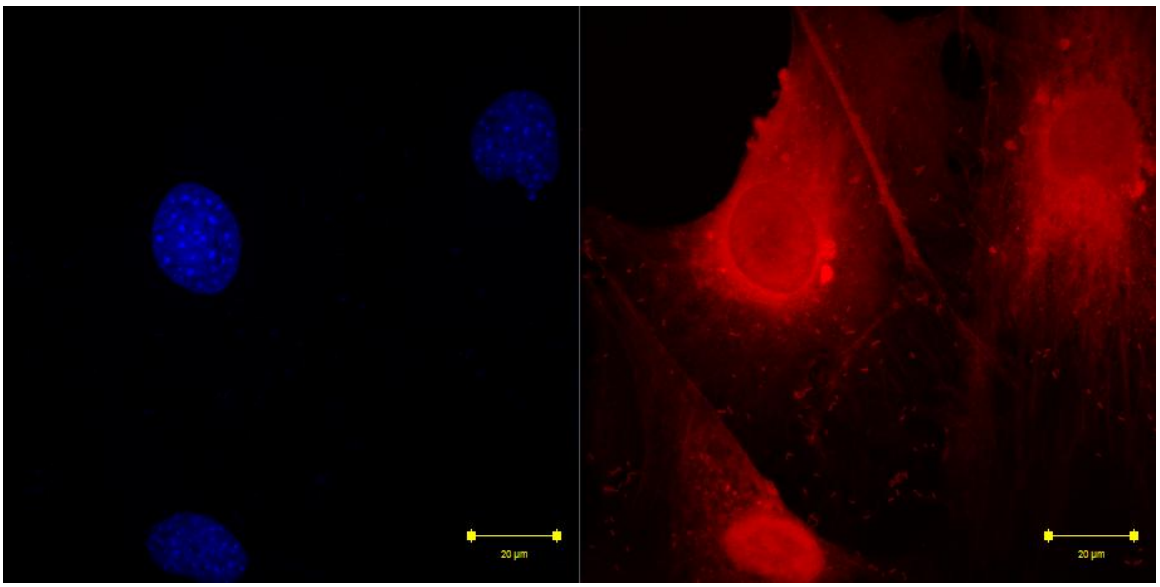


Figure 30: representative image of Senescent MTF. On the left, DAPI counter stains the DNA. On the right, NanoOrange stains cellular proteins. [bar = 20µm]

Staining of mouse tissues

Lung

Samples from 6 young (5 months old) and 6 old (36 months old) animals were embedded in OCT and cut in cryostat at Chamber Temperature -29°C and a Object Temperature of -27°C.

As for all the other tissue experiments, the 7 µm slices were melted on a poly-lysine covered coverslip and immediately fix with 4% paraformaldehyde, 0.5% Triton X-100 at Room Temperature for exactly 20 minuntes. Coverslips were then washed three times in PBS-T 0.2% and the staining protocol was carried over as described in the paper.

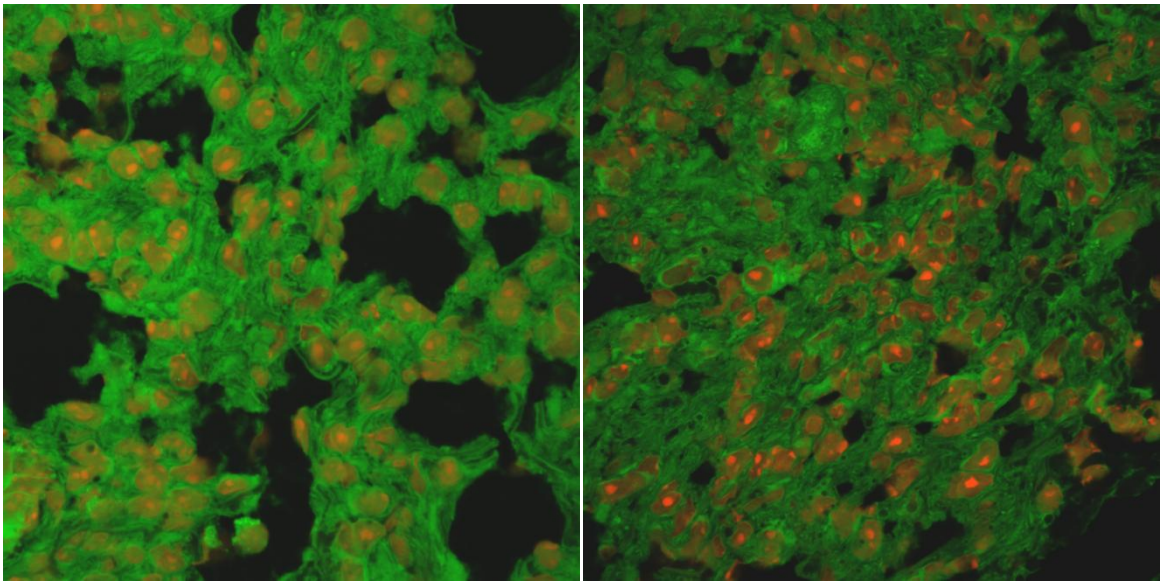


Figure 31: representative images of 5 mo mouse lung stained for total protein (green in the confocal's pseudocolors) and counterstained for DNA (red in confocal's pseudocolors).

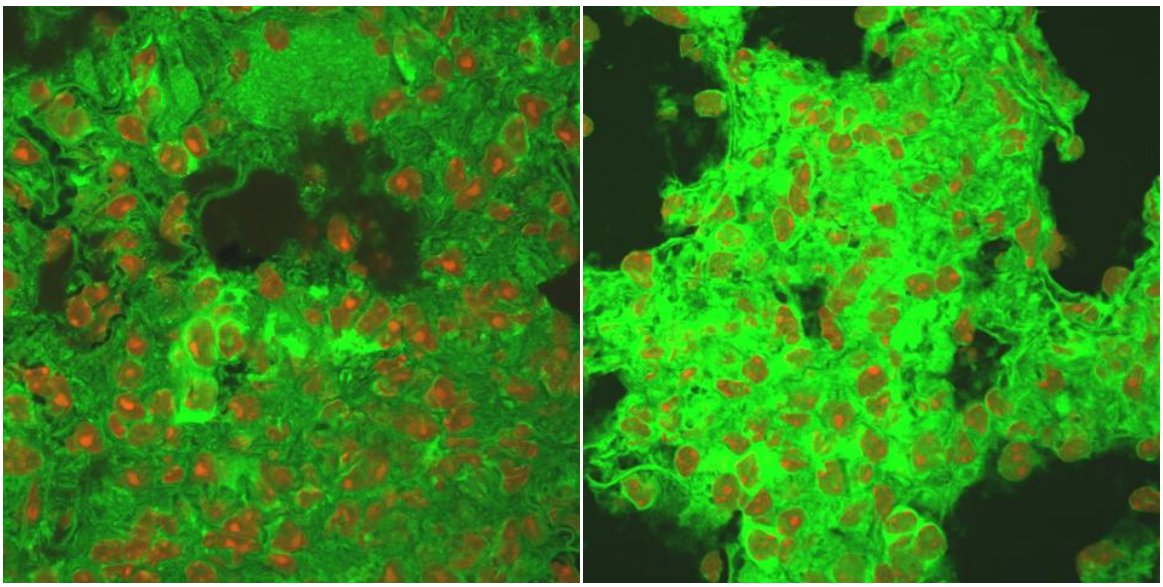


Figure 32: representative images of 36 mo mouse lung stained for total protein and counterstained for DNA.

Liver

Samples were processed in the exact same way as lung, picking 6 young and 6 old animals. In this case the cutting temperature in the cryostat was -19°C as Chamber Temperature and -17°C as Object Temperature.

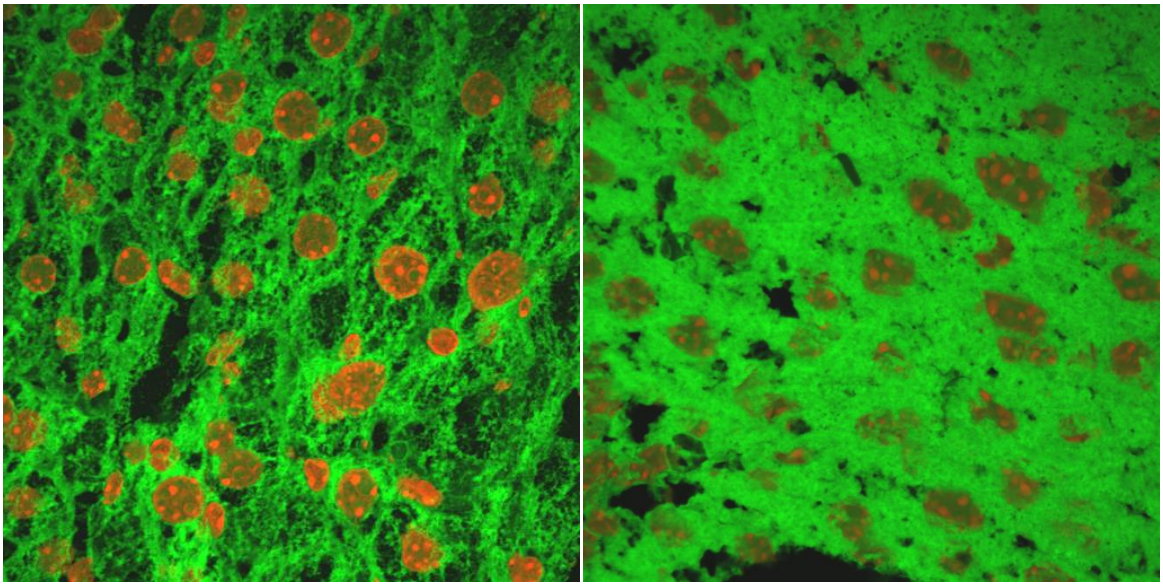


Figure 33: representative images of 5 mo mouse liver stained for total protein (green) and counterstained for DNA (red).

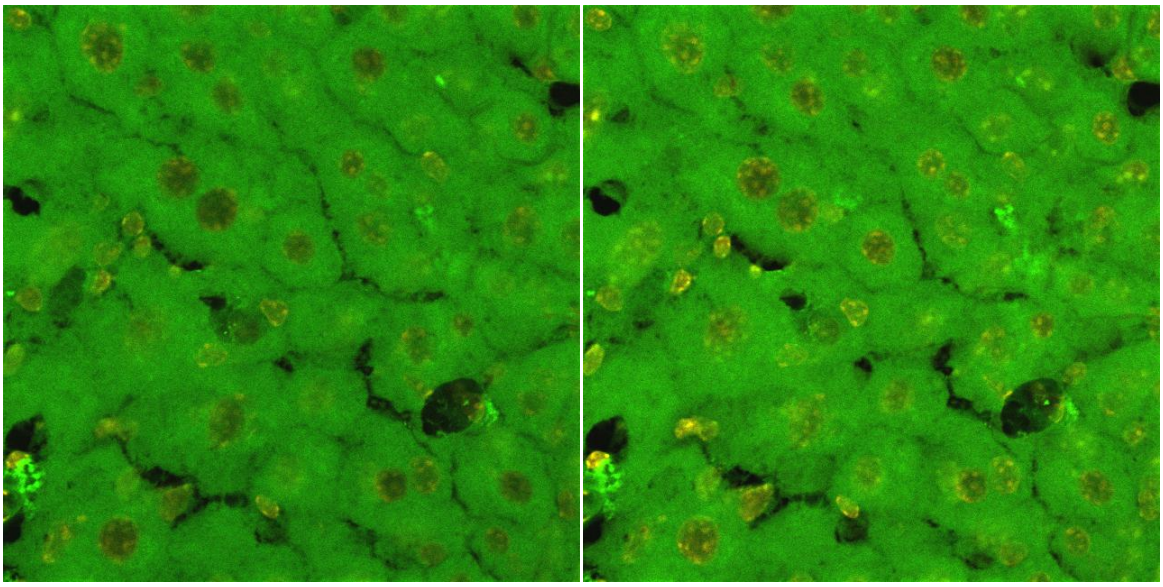


Figure 34: representative images of 36 mo mouse liver stained for total protein (green) and counterstained for DNA (red).

Kidney

Samples were processed in the exact same way as liver and lung, with the same 6 young and 6 old animals. In this last case, the cutting temperature in the cryostat was -23°C as Chamber Temperature and -21°C as Object Temperature.

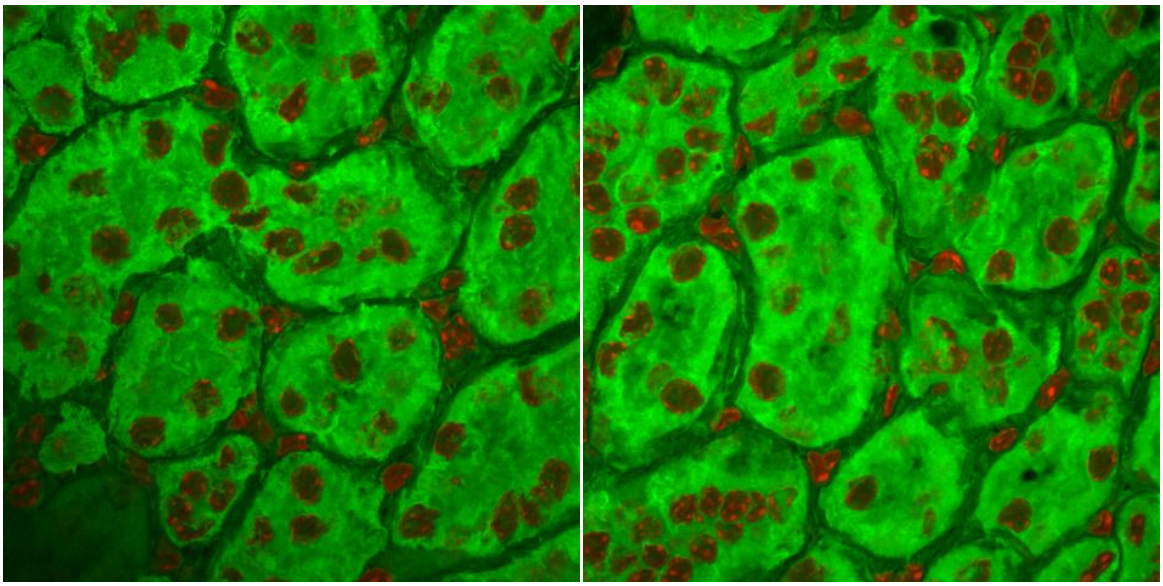


Figure 35: representative images of 5 mo mouse kidney stained for total protein (green) and counterstained for DNA (red).

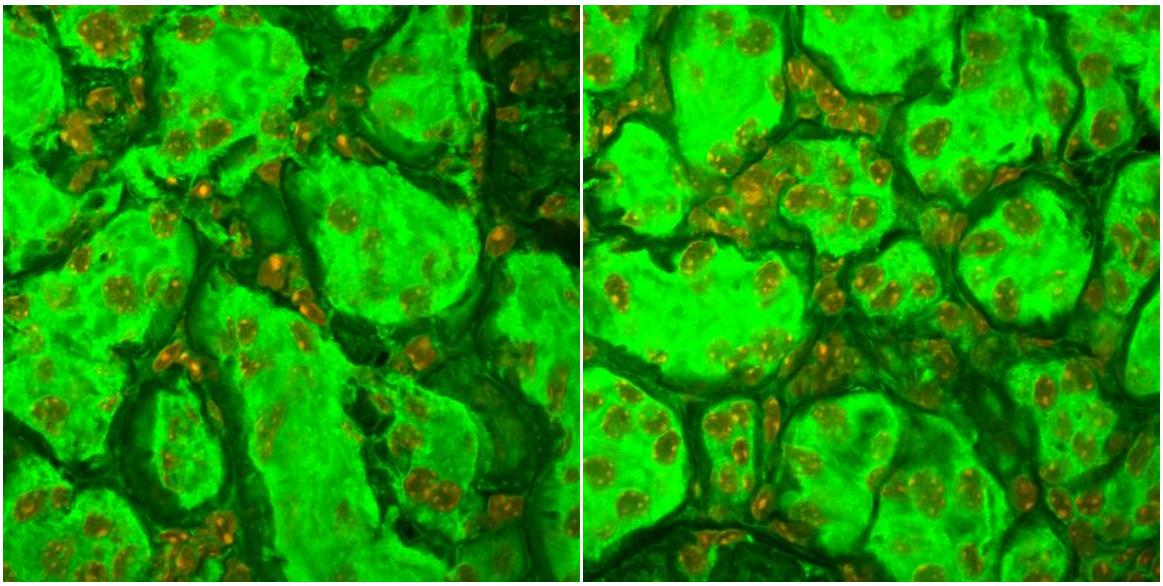


Figure 36: representative images of 36 mo mouse kidney stained for total protein (green) and counterstained for DNA (red).

All the images obtained have been processed and quantify for nuclear protein content and several other feature that may become crucial in further models.

Remarks

Overall the data that haven't been published in the paper, especially the ones from tissues are very solid and coherent among them. They will be absolutely kept in high regard when planning further experiments on this newly developed biomarker as it is the nuclear protein content. Our knowledge in terms of nuclear proteins will soon be expanded with more tissues, like as brain, muscle and spleen; this is going to end in a better definition of the nuclear complexity *in vivo* and in a very important database of results to correlate with parallel specific information.

Appendix 2 - Published data

We have developed an easy and flexible method, based on the merocyanine dye known as NanoOrange, to visualize and quantitatively measure total protein levels by high resolution fluorescence microscopy. In the paper we published the results of NanoOrange stainings, plus their combination with antibody-based immunofluorescence, thus providing both specific target and total protein information in the same specimen. Combining the method with automated image analysis platforms it was possible to run high throughput analysis.

We document here increasing protein content and density in nuclei of senescent human and mouse fibroblasts in vitro, and in liver nuclei of aged mice in vivo.

Article:

Nuclear protein accumulation in cellular senescence and organismal aging revealed with a novel single-cell resolution fluorescence microscopy assay.

De Cecco M, Jeyapalan J, Zhao X, Tamamori-Adachi M, Sedivy JM.

Aging (Albany NY). 2011 Oct;3(10):955-67.

PMID: 22006542

URL: http://www.ncbi.nlm.nih.gov/pubmed/22006542

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Research Paper

Nuclear protein accumulation in cellular senescence and organismal aging revealed with a novel single-cell resolution fluorescence microscopy assay

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Running title: *Increase of Total Protein Content in Senescent Cells*
Key words: *Aging, cellular senescence, quantitative protein assay, fluorescence microscopy, NanoOrange® reagent*

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Abstract: Replicative cellular senescence was discovered some 50 years ago. The phenotypes of senescent cells have been investigated extensively in cell culture, and found to affect essentially all aspects of cellular physiology. The relevance of cellular senescence in the context of age-associated pathologies as well as normal aging is a topic of active and ongoing interest. Considerable effort has been devoted to biomarker discovery to enable the microscopic detection of single senescent cells in tissues. One characteristic of senescent cells documented very early in cell culture studies was an increase in cell size and total protein content, but whether this occurs in vivo is not known. A limiting factor for studies of protein content and localization has been the lack of suitable fluorescence microscopy tools. We have developed an easy and flexible method, based on the merocyanine dye known as NanoOrange, to visualize and quantitatively measure total protein levels by high resolution fluorescence microscopy. NanoOrange staining can be combined with antibody-based immunofluorescence, thus providing both specific target and total protein information in the same specimen. These methods are optimally combined with automated image analysis platforms for high throughput analysis. We document here increasing protein content and density in nuclei of senescent human and mouse fibroblasts in vitro, and in liver nuclei of aged mice in vivo. Additionally, in aged liver nuclei NanoOrange revealed protein-dense foci that colocalize with centromeric heterochromatin.

INTRODUCTION

Normal somatic cells, with the exception of germ cells and some stem cells, display a finite replicative capacity. This phenomenon was first described in cultured human fibroblasts [1], and is commonly referred to as replicative cellular senescence. Subsequently, these observations were extended to a wide variety of vertebrate species and cell types, and senescence is now believed to be a general property of replicative cells [2]. Cellular senescence has been studied predominantly in cell culture (in vitro), although evidence is accumulating that this process also occurs in intact organisms (in vivo) under a variety of pathological and normal conditions [3-5]. A well studied trigger of cellular senescence is the shortening of telomeres [6], but many other stimuli, most importantly the activation of oncogenes and a variety of genotoxic stresses, have also been documented [7]. The phenotypes of senescent cells affect most, if not all aspects of cellular physiology, including gene expression, chromatin organization, protein processing and metabolism [3, 8]. Given the extensive, complex, and often cell-type specific nature of these changes this remains an active area of investigation.

In vivo studies have been especially hampered by the complex phenotypes of senescent cells as well as their low abundance under most normal conditions. We, as well as others, have expended considerable effort in developing biomarkers to enable the microscopic detection of single senescent cells in tissues [9-11].

Bibliography

1. Bayreuther K, Francz PI, Gogol J, Hapke C, Maier M, Meinrath HG. Differentiation of primary and secondary fibroblasts in cell culture systems. Mutat Res. 1991 Mar-Nov;256(2-6):233-42. Review. PubMed PMID: 1722013.
2. Cesur N, Apak TI, Aboul-Enein HY, Ozkirimli S. LC determination of aminoglutethimide enantiomers as dansyl and fluorescamine derivatives in tablet formulations. JournalJ Pharm Biomed Anal (2002) 28:487-492ID:PN46793.
3. Cristofalo VJ, Pignolo RJ (1993) Replicative senescence of human fibroblast-like cells in culture. Physiol. Rev. 73, 617–638.
4. Dell'orco RT, Guthrie PL. Altered protein metabolism in arrested populations of aging human diploid fibroblasts. Mech Ageing Dev. 1976;5(6):399-407. PubMed PMID: 994559.
5. Go K, Horikawa Y, Garcia R, Villarreal FJ. Fluorescent method for detection of cleaved collagens using O-phthaldialdehyde (OPA). J Biochem Biophys Methods (2008) 70:878-882.
6. Graves SW, Woods TA, Kim H, Nolan JP. Direct fluorescent staining and analysis of proteins on microspheres using CBQCA. Cytometry A. 2005 May;65(1):50-8.
7. Hapuarachchi S, Aspinwall CA. Design, characterization, and utilization of a fast Fluorescence derivatization reaction utilizing o-phthaldialdehyde coupled with fluorescent thiols. J Electrophoresis. 2007 28:1100-1106ID:PN58768.
8. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res 1961; 25:585-621.
9. Hayflick L. Aging human cells. Triangle. 1973;12(4):141-7. PubMed PMID: 4769081.
10. Hayflick L. Human cells and aging. Sci Am. 1968 Mar;218(3):32-7.
11. Huang Z, Park JI, Watson DS, Hwang P, Szoka FC Jr. Facile synthesis of multivalent nitrilotriacetic acid (NTA) and NTA

conjugates for analytical and drug delivery applications. Bioconjug Chem. 2006 Nov-Dec;17(6):1592-600.

12. Jones LJ, Haugland RP, Singer VL. Development and characterization of the NanoOrange protein quantitation assay: a fluorescence-based assay of proteins in solution. Biotechniques. 2003 Apr;34(4):850-4, 856, 858 passim. PubMed PMID: 12703310.
13. Jun JI, Lau LF. Cellular senescence controls fibrosis in wound healing. Aging (Albany NY) 2011; 2:627-31.
14. Kaftory A, Hershko A, Fry M. Protein turnover in senescent cultured chick embryo fibroblasts. J Cell Physiol. 1978 Feb;94(2):147-60. PubMed PMID: 621215.
15. Kagan VE, Gleiss B, Tyurina YY, Tyurin VA, Elenström-Magnusson C, Liu SX, Serinkan FB, Arroyo A, Chandra J, Orrenius S, Fadeel B. A role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. JournalJ Immunol (2002) 169:487-499ID:PN52258.
16. Nardella C, Clohessy JG, Alimonti A, Pandolfi PP. Pro-senescence therapy for cancer treatment. Nat Rev Cancer 2011; 11:503-11.
17. Noble JE, Bailey MJ. Quantitation of protein. Methods Enzymol 2009; 463:73-95.
18. Sedivy JM, Munoz-Najar UM, Jeyapalan JC, Campisi J. Cellular senescence: A link between tumor suppression and organismal aging? In: Guarente L, Partridge L, eds. The Molecular Biology of Aging. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 2008.
19. Yamamoto T, Kozasa T, Aoki H, Sekiguchi H, Morino S, Nakamura S. Genomic analyses of bovine viral diarrhea viruses isolated from cattle imported into Japan between 1991 and 2005. Vet Microbiol. 2008 Mar 18;127(3-4):386-91. Epub 2007 Aug 19.