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# Exploring the role of SUMOylation in cellular transformation and colorectal cancer

Eleftheria Chalatsi

► **To cite this version:**

Eleftheria Chalatsi. Exploring the role of SUMOylation in cellular transformation and colorectal cancer. Cancer. Université Pierre et Marie Curie - Paris VI, 2017. English. NNT : 2017PA066273 . tel-02298577v2

**HAL Id: tel-02298577**

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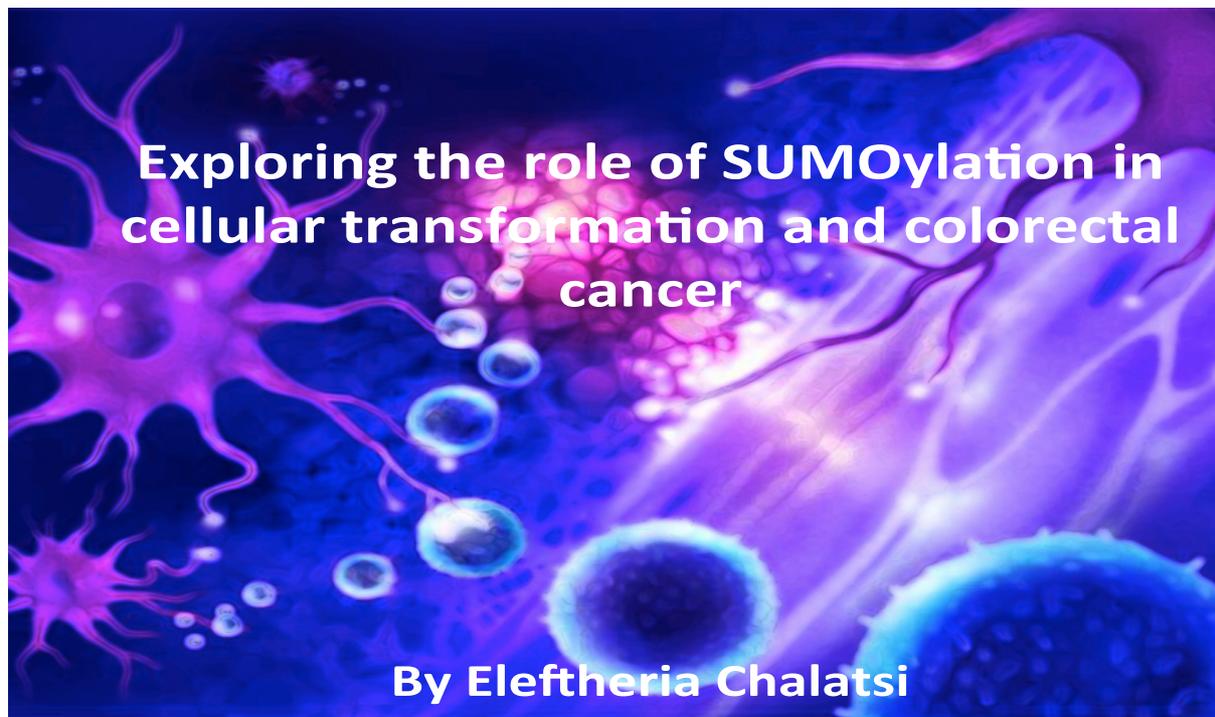
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# University Pierre and Marie Curie

Doctoral school : Complexité Du Vivant - ED515

*Nuclear Organization and Oncogenesis Unit (INSERM U993)*



Doctoral thesis of Cancer biology and Immunology, Cancer therapeutics

Supervised by Anne Dejean

Presented and defended publicly on September 25th 2017

In front of a jury composed of:

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Dr. Dimitris XIRODIMAS	CNRS research director	Reviewer
Dr. Silvia FRE	INSERM CR1	Examinator
Dr. Guillaume BOSSIS	CNRS CR2	Examinator
Dr. Frederic DEVAUX	UPMC university professor	President
Prof. Anne DEJEAN	INSERM research director	Thesis Director (Invited member)



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Σα βγεις στον πηγαιμό για την Ιθάκη, να εύχεται να ναι μακρύς ο δρόμος, γεμάτος περιπέτειες,  
γεμάτος γνώσεις.

....

Πάντα στον νου σου νάχεις την Ιθάκη. Το φθάσιμον εκεί είν' ο προορισμός σου. Αλλά μη  
βιάζεις το ταξείδι διόλου. Καλλίτερα χρόνια πολλά να διαρκέσει· και γέρος πια ν' αράξεις στο  
νησί, πλούσιος με όσα κέρδισες στον δρόμο, μη προσδοκώντας πλούτη να σε δώσει η Ιθάκη.

Η Ιθάκη σ' έδωσε τ' ωραίο ταξείδι. Χωρίς αυτήν δεν θά βγαινεις στον δρόμο. Αλλά δεν έχει να  
σε δώσει πια.

Κι αν πτωχική την βρεις, η Ιθάκη δεν σε γέλασε. Έτσι σοφός που έγινες, με τόση πείρα, ήδη θα  
το κατάλαβες η Ιθάκες τι σημαίνουν.

*As you set out for Ithaka hope the voyage is a  
long one, full of adventure, full of discovery.*

....

*Keep Ithaka always in your mind. Arriving  
there is what you are destined for. But do not  
hurry the journey at all. Better if it lasts for  
years, so you are old by the time you reach the  
island, wealthy with all you have gained on the  
way, not expecting Ithaka to make you rich.*

*Ithaka gave you the marvelous journey.  
Without her you would not have set out. She  
has nothing left to give you now.*

*And if you find her poor, Ithaka won't have  
fooled you. Wise as you will have become, so  
full of experience, you will have understood by  
then what these Ithakas mean.*

*Quand tu prendras le chemin d'Ithaque,  
souhaite que la route soit longue, pleine  
d'aventures, pleine d'enseignements.*

....

*Garde toujours Ithaque présente à ton  
esprit. Y parvenir est ta destination finale.*

*Mais ne te hâte surtout pas dans ton  
voyage. Mieux vaut le prolonger pendant  
des années ; et n'aborder dans l'île que  
dans ta vieillesse, riche de ce que tu auras  
gagné en chemin, sans attendre d'Ithaque  
aucun autre bienfait.*

*Ithaque t'a offert ce beau voyage. Sans  
elle, tu n'aurais pas pris la route. Elle n'a  
rien de plus à t'apporter.*

*Et même si elle est pauvre, Ithaque ne t'a  
pas trompé. Sage comme tu l'es, avec une  
expérience pareille, Tu as sûrement déjà  
compris ce que les Ithakas signifient.*

## Acknowledgements

I would like to begin by thanking all the members of the jury: Dominique Leprince, Dimitris Xirodimas, Laurence Levy, Silvia Fre and Frederic Devaux who, despite a very busy schedule took the time to take part in this thesis defense jury.

I would like to thank Anne Dejean for accepting me in her laboratory after a skype-call four years ago. Merci à vous, Anne, for the confidence and trust you placed in me, providing me with complete independence in an outstanding laboratory with valuable collaborations and the best working conditions possible. Thank you for your support and for being an inspiration for women in science, proving that with hard work and dedication both professional and personal success is possible.

I would like to thank all the current and past members of the ONO team. The multitude of incredible individuals I was able to meet and collaborate with throughout the years warrant much more than a few lines of acknowledgement.

Being able to interact and exchange with the different personalities and cultures from over 14 countries, all working together in harmony, has been one of the most enriching and rewarding experiences of my career in science and an unbelievable learning opportunity.

Merci Alex first of all for your patience with my French, but most importantly with the indispensable help throughout the thesis, we would all be lost without you. Merci à toi, Sophie, pour avoir pris le relais. Mes remerciements à Pascal for being always there for discussions and advice whether work related or of the more politico-philosophical persuasion. Merci à toi Thibaut for being the most loyal “coffee break” partner, friend and confidant throughout all these years, and of course Pablo and Elena for also always being there to listen to the frustrations of mad Greek scientist.

Pablo and Ying, your advice and technical support were invaluable to this process and achievement; I don't know how I would have made it without you. I would remiss if I did not also thank you, Jacob, for your constructive input. Oliver and all the members of your team for the scientific input and the good times shared at our “meeting of the minds” during our afterwork beer hours, but in particular Gregory for being there in each MUSE frustration and PEF, the bioinformatician genius, for helping me where I was completely lost. Jack and Ilan I must also acknowledge your communicative sense of humor, for all the laughs and good times and scientific exchanges. Merci à toi, Agnes, for being always there to help me and for

being the generous and attentive person that you are. Gracias a ti, Juan Pablo, por tu fiel amistad, tu apoyo en la bioinformática y los consejos preciosos que me diste cuando preparaba mi aventura peruana. Victoria, our latest member, welcome and all the best for the adventure you are about to start. Florence for all the administrative support, your vivid personality and our conversations making everyday more interesting and spicy. Nacho, I couldn't wish for a better person for the continuation of my work, I am happy we got to collaborate even for such a short period, thank you for all your input and help. Adrien, Pierre and Helene for accompanying me in my first steps in the lab. Cathy and Monique for making the lives of all of us easier with your precious-behind the spotlight- help.

I would like to thank also people from the other teams on the floor and not only for enriching this scientific journey. In particular, one of the sweetest persons on this planet, my first PI, Prof. Penelope Mavragani for making my first lab experience so special and beautiful, as well as Prof. Antoniewski for opening his lab to me and bringing me to Paris and Clement Carré for his valuable supervision at the time and help throughout the years.

The challenges faced during this journey to PhD completion were only further enriched by a personal adventure that has involved 4 countries in 3 different continents.

To this end, I would also like to thank all those who made the Elma outside of the lab laugh, learn and ultimately thrive through their love, support, kindness, patience and above all taste for adventurous madness that made all of this come together.

Thank you to my friends here and in Greece, in particular Filitsa for being there my whole life; Natalia, such a nice surprise finding out of the blue a friend and support system for life; Alexandra for the long friendship from Thessaloniki to Paris and Pasteur, thank you for your support during the reduction of my thesis; Stratos for believing in me and all your nice words in moments of doubt. I am also grateful for the people I met in Papua New Guinea, cheering for me and giving me extra strength to finish this manuscript.

I would like to thank my parents, grandparents and brother for providing me throughout the thesis and my life in general with patience, understanding and unwavering supporting for my professional and personal goals even when that translated to such few time spent together throughout these past 4 years. But most importantly, to my parents, thank you for the sacrifices you made. You have always put me and my brother as your absolute

priority, providing us with all the weapons and strength necessary to make it here. Thank you for encouraging me to reach for the stars and chase my dreams. Whatever I am or hope to be I owe it to you.

I would like to express my gratitude to my belle-famille en or that embraced me and provided me with moral and emotional support. From the parents, to the siblings and significant others, to grandmothers and of course the Reine Monard. They accepted without a doubt and put their heart into helping mould this improbable relationship, sacrificing the precious time they could have with their kid and sharing it with me and my family. I am incredibly fortunate to have all of you in my life and I will be forever grateful for all the support.

I would like to thank my partner in crime, the love of my life, Armand. First of all, for being patient and crazy enough to proof-read this manuscript. But most importantly, for his support throughout this journey, the strength he has given me to continue, the endless hours of trips he went through to come back to me, with all the sacrifices that this required, working with me to write our story in seemingly impossible conditions. So many challenges, and yet, we made it all work. Thank you for your support.

For anything worth having one must pay the price; and the price is always work, patience, love, self-sacrifice. This is all this thesis is about and it is only the beginning of my journey. Cheers to a new chapter in the book of life.

## **Collaborations**

Because this work is also the result of a collaborative effort, I would like to thank our collaborators for their help by providing their expertise and tools to this project.

Gregory Jouvion, from the team “Human Histopathology and Animal Models” of the Pasteur Institute for his important help with the histopathological analysis of samples.

Jacco Van Rheenen and his lab and in particular Saskia Ellenbroek for their valuable help in the analysis of the Lgr5+ stem cells, and their helpful discussions and advices.

Sean Kennedy and the Biomics team of Pasteur Institute for the help with microbiota sequencing and analysis.

Christelle Thibault-Carpentier from the Genomeast platform of the IGBMC for the help with one of the affymetrix experiments.

Giulia Nigro and Philippe Sansonetti’s lab for the scientific discussions and the Lgr5 mouse.

All the people and scientists who I had the chance to encounter at the Pasteur Institute and in conferences, which led to discussion allowing to further this project.

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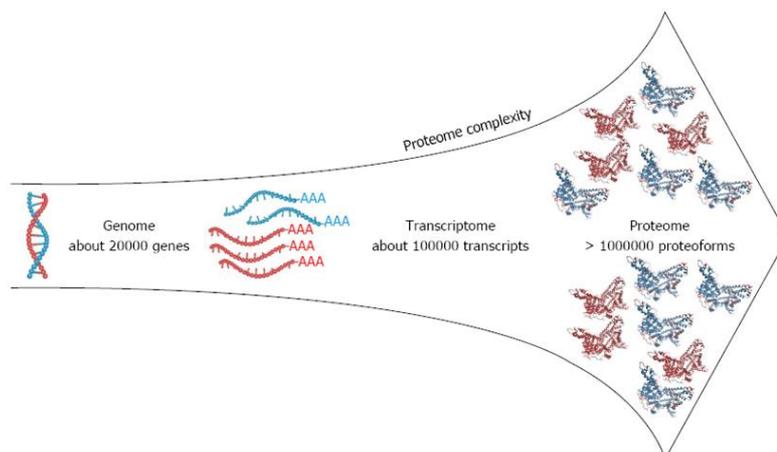
## List of abbreviations

4OHT 4-hydroxytamoxifène  
AOM Azoxymethane  
APL Acute Promyelocytic Leukemia  
C-MYC Myelocytomatosis virus oncogene cellular homolog  
CRC Colorectal Carcinoma  
DMEM Dulbecco's Modified Eagle Medium  
DNA DeoxyriboNucleic Acid  
DSS Dextran Sodium Sulfate  
DUBs DeUBiquitinating enzyme  
GFP Green Fluorescent Protein  
KD Knock-down  
KO Knock-out  
Lgr5 Leucine-rich repeat-containing G-protein coupled receptor 5  
MEF Mouse Embryonic Fibroblast  
mRNA messenger RNA  
NFKB nuclear factor kappa b  
PBS Phosphate Buffered Saline  
PIAS Protein Inhibitor of Activated STATs  
PML ProMelocytic Leukemia protein  
PML-NBs PML Nuclear Bodies  
PTMs Post Translational Modifications  
RNA Ribonucleic acid  
RT QPCR Reverse-transcription - quantitative Polymerase Chain Reaction  
SENP Sentrin Protease  
SIM SUMO - interacting domain  
STAT Signal Transduced Activation Transcription  
SUMO Small Ubiquitin-like Modifier  
TGF- $\beta$  Transforming growth factor beta  
TF Transcription Factor  
TSS Transcription Start Site  
TTS Transcription Termination Site  
UBC9 Ubiquitin Conjugating 9  
UBL Ubiquitin-like  
WT Wild-type

# **INTRODUCTION**

## Post-translational modifications

One of the greatest research accomplishments that marked the 21st century was the completion of the sequencing of the human genome which revealed that it is comprised of between 20,000 and 25,000 genes (International Human Genome Sequencing Consortium, 2004). This number is surprisingly low for such a complex life form, while the total number of proteins in the human proteome is estimated at over 1 million (Jensen, 2004). Over the past few decades, it became more and more clear that the human proteome is vastly more complex than the human genome. Single genes encode multiple proteins and mechanisms such as genomic recombination, transcription initiation at alternative promoters, differential transcription termination, and alternative splicing of the transcript, generate different mRNA transcripts from a single gene (Ayoubi and Van De Ven, 1996). After translation most proteins are covalently modified at least once by another mechanism, the post-translational modification of proteins (PTMs) adding a layer of complexity between the level of the genome to the proteome (Figure 1).



**Figure 1 Proteome complexity.** Changes at the transcriptional and mRNA levels increase the size of the transcriptome relative to the genome, and the various different post-translational modifications exponentially increase the complexity of the proteome relative to both the transcriptome and genome (Bennike et al., 2014).

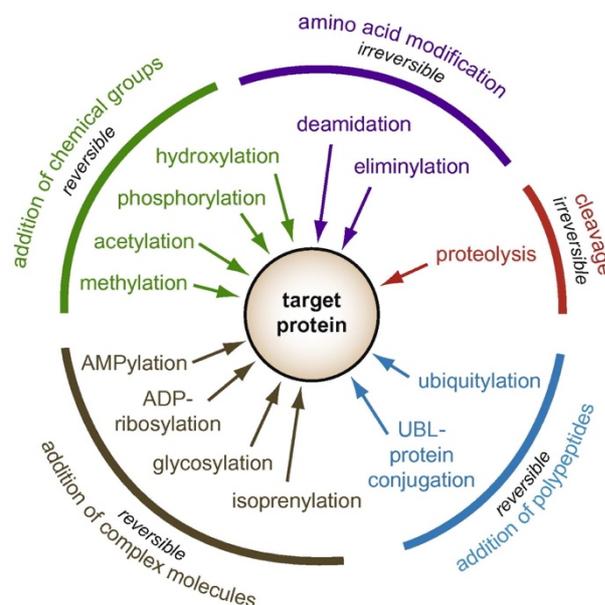
PTMs are modifications that occur on a protein catalyzed by enzymes after its translation by ribosomes is complete. PTMs have emerged as crucial molecular regulatory mechanisms that control the specificity, timing, duration and amplitude of virtually all physiological processes as they regulate the activity, localization and interaction with other

cellular molecules such as proteins, nucleic acids, lipids and cofactors, playing a key role in functional proteomics.

The objective of this thesis is to evaluate the impact of the dysregulation of one post-translational modification of proteins, the sumoylation, in the pathophysiological process of cancer, notably focusing on intestinal and colon cancer as well as cellular transformation and tumorigenesis. For this purpose, in this manuscript we will first discuss the different roles that SUMOylation plays in pathways involved directly and indirectly in cancer and we will eventually focus on the role of SUMO in cancer.

## Biochemical diversity of post-translational modifications

There is a wide range of post-translational modifications available to the cell to diversify its proteome. They represent the major level of regulation, from very fast and reversible to slow or irreversible processes (figure 2). Currently more than 450 different post-translational modifications are listed in the Uniprot database (Consortium, 2012). They consist in the chemical modification of proteins by the addition of chemical groups or polypeptides, targeting 15 different of the 21 amino acids typically found in eukaryotes, or the cleavage of the precursors (Ribet and Cossart, 2010).



**Figure 2 Diversity of post-translational modifications.** A protein can be modified in a reversible manner by addition of a chemical group, complex molecules or polypeptides, or in an irreversible manner by cleavage or subtraction of chemical groups of their amino-acids (Ribet and Cossart, 2010).

Proteins can be modified multiple times at different sites with different modifications, each of them having a possible impact on future or existing modifications. Moreover, some modifications are targeting the same residue on the same protein leading to post-translational modification competition. Every modification is capable of influencing others, thereby creating crosstalks. In the past few years, evidence for extensive crosstalk between PTMs has grown, with the competitive interplay between O-GlcNac and O-phosphorylation being one of the first and best-established examples (Hart *et al.*, 1995). Positive and negative crosstalk can also be distinguished.

In positive crosstalk, the initial PTM serves as an active trigger for the addition or removal of a second PTM, or as a recognition site for other proteins, as for instance in phosphorylation-dependent ubiquitination (Koepp *et al.*, 2001; Nguyen, Kolch and Kholodenko, 2013) and SUMOylation (Hietakangas *et al.*, 2006). In contrast, negative crosstalk can include direct competition of two PTMs for the same amino acid or indirect effects when a specific PTM masks the recognition site for second PTM, thus preventing its addition and/or removal (Hunter, 2007). This crosstalk between PTMs and the potential existence of a so-called “PTMcode,” (Lothrop, Torres and Fuchs, 2013) regulating protein functions and cellular signaling, represent just another level of complexity and pose a major challenge to ongoing and future PTM research.

In addition, these modifications can also for the most part be removed by specialized enzymes. This multitude of modification with infinite combinations and reversibility is a major key to understanding cellular life as they affect protein function. Therefore, any conditions impairing this key ecosystem could lead to diseases such as cancer.

## **Ubiquitination**

The Ubiquitination was the first post-translational modification ever described. It is the post-translational modification process by which ubiquitin is attached via an isopeptide bond to lysine residues on a protein.

Initially discovered by Goldstein *et al.* in 1975 (Goldstein *et al.*, 1975) it was only in the early 80s that the works of Hershko, Ciechanover and Rose permitted the characterization of the complex enzymatic cascade that allows this modification. These works led to the

association of the poly-ubiquitination of the proteins with their degradation by the proteasome, leading to the Nobel Prize of Chemistry in 2004 (Hershko, Ciechanover and Rose, 1979, 1981; Hershko *et al.*, 1980). From that discovery, it became apparent that the ubiquitination has a multitude of functions in the cell other than the homeostasis of proteins. Ubiquitin-dependent protein degradation is involved in the regulation of many important and diverse proteins in the cell.

The Ubiquitin (Ub) is present in abundance inside all the cells of the organism. Present in all eukaryotic organisms studied, 4 different genes (*Ubb*, *Ubc*, *Uba52*, *Rps27a*) encode for this protein in all of those organisms, reflecting its importance in the life of the eukaryotic cell. In mice and in humans, the monomeric Ub-ribosomal fusion genes, *Uba52* and *Rps27a*, encode fusions between Ub and two small ribosomal proteins and are expressed consecutively. The polyubiquitin genes *Ubb* and *Ubc* encode for the poly-proteins of ubiquitin and have long been known to play a role in stress-response and induced when cells are exposed to threats such as heat shock, starvation, DNA damaging agents, proteasome inhibitors, oxidative stress and so on (Finley, Özkaynak and Varshavsky, 1987; Finley, Bartel and Varshavsky, 1989; Fornace *et al.*, 1989; Baker and Board, 1991; Finch *et al.*, 1992; Vihervaara *et al.*, 2013)(15) Baker and Board, 1991 Finley et al., 1987, 1989; Fornace et al., 1989; Finch et al., 1992 ; Vihervaara et al., 2013).

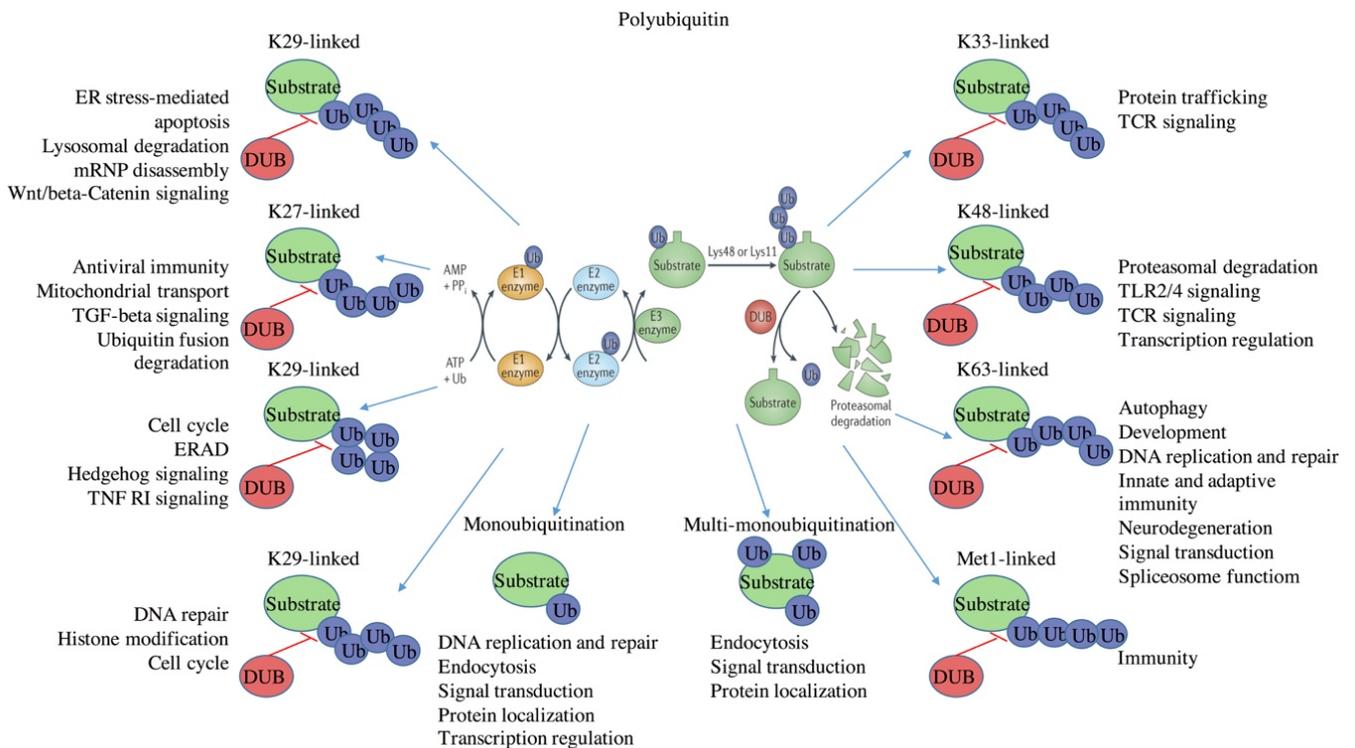
Although *Ubb* and *Ubc* are transcriptionally upregulated in response to cell stress, studies have shown the importance of these genes to basal ubiquitin levels, as their genetic deletion proved lethal in embryonic level for *Ubc* (Ryu *et al.*, 2007) or caused infertility and adult-onset hypothalamic neurodegeneration with metabolic and sleep abnormalities (Ryu *et al.*, 2008).

The ubiquitination consists of three steps: activation by ubiquitin-activating enzymes (E1 enzymes), conjugation by ubiquitin-conjugating enzymes (E2s) and attachment to the substrate protein by ubiquitin ligases (E3s) (Hershko and Ciechanover, 1998).

The cascade of ubiquitination is initiated with the maturation of the ubiquitin thanks to the cleavage of its C-terminal domain, exposing a glycine residue in the mature form. The E1 enzyme (*Ube1* in mice and humans) is the activating enzyme. It forms a thioester bond between its internal Cys residue and the carboxy-terminal glycine of ubiquitin in an ATP-



the polyubiquitination in the form of ubiquitinating chains that is greatly increasing its versatility. Indeed, has been established that the linkage involved in the degradation from the proteasome is in the lysine 48 (K48). The different linkage forms confer a multitude of functions which are reported below (figure 4).



**Figure 4 Functional importance of the different ubiquitin linkage types.** Ubiquitin can be attached to substrate proteins in three different manners: monoubiquitination, multi-monoubiquitination, and polyubiquitination. Eight residues within ubiquitin can be utilized to form polyubiquitin chains [K6, K11, K27, K29, K33, K48, K63, and Met1 (linear)] and linkage type directs the modified proteins to different cellular fates. Polyubiquitin chains of all linkages listed above have been detected *in vivo* and have been shown to differentially affect many cellular processes, signaling pathways, and disease states.

The need to specifically target a broad array of substrates accounts for the great diversity among the estimated >600 human E3s. Nonetheless, E3 ligases are classified in three families based on their structural domains and the mechanism of action:

## RING

The E3 ligases of the RING family consist of a zinc-binding domain called RING (Really Interesting New Gene) or by a U-box domain and are the most abundant type. These ligases catalyze the direct transfer of the ubiquitin from the cysteine of the E2 to the lysine of the target protein. They can function as monomers, homodimers binding two E2s or heterodimers.

## HECT

The E3 ligases of the HECT (homologous to the E6AP carboxyl terminus) domain family catalyze the transfer of ubiquitin through a two-step reaction where ubiquitin is first transferred to a cysteine on the E3 and then to the substrate. The HECT domain is at the C-terminal part of the ligase and is involved in the catalysis while the N-terminal part of the ligase is responsible for substrate specificity. Based on their N-terminal extensions, we have three different subfamilies of human HECTs: the Nedd4 family, the HERC (HECT and RCC1-like domain) family and other HECTs that contain various domains.

## RBR

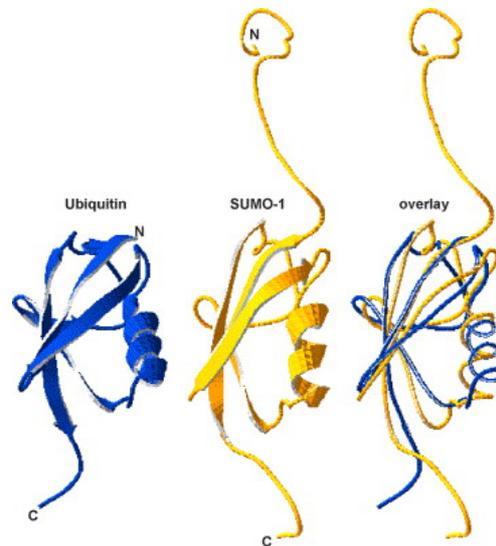
The E3 ligases of RBR (RING-betweenRING-RING) domain family catalyze ubiquitin transfer the same way as HECT ligases, through the two-step reaction. As their name indicates, the RBR ligases consist of two RING domains, RING1 and RING2, separated by an in-between-RING domain (IBR). RING1 recruits the ubiquitin-charged E2, approaches it to the RING2 domain provoking the transfer of ubiquitin to the RING2 which then transfers the ubiquitin to the substrate.

The ubiquitination and the associated proteolysis are involved in numerous pathologies and have been the focus of numerous studies that in certain cases have shown a clinical utilization. It is notably the case of the inhibitor of the proteasome bortezomib, used in clinical cases against the multiple myeloma and the mantle cell lymphoma. The implication of the ubiquitination in key cellular processes such as the apoptosis or the inflammation makes it a principle target for the fight against the inflammatory diseases, the neurodegenerative diseases and certain cancers (Vucic, Dixit and Wertz, 2011).



As we can see in Table 1, some UBLs such as Rub1/NEDD8 display a high degree of sequence similarity to ubiquitin whilst others such as SUMOs do not. Due to their role in various processes of the eukaryotic cell however, the UBLs proteins that have been more intensively studied and also feature the most known substrates are those of the SUMO (Small Ubiquitin-like MOdifier) family.

SUMO is a peptide of 10kDa, which has very similar three-dimensional structures despite its relatively low sequence identity with ubiquitin (~15%), as shown below (Figure 5). As demonstrated in Figure 5, the SUMO peptide features a protruding long and flexible N-terminal domain, which is absent in ubiquitin.



**Figure 5 Comparison of the structures of ubiquitin and SUMO-1 in humans.** Both structures share the tightly packed secondary structure made of  $\alpha$ -helices and  $\beta$ -sheets along with the di-glycine motif at the C-terminus. A long flexible chain at the N-terminus is only observed in SUMO (Dohmen 2004).

## **SUMOylation**

### **Discovery of the modification of proteins by SUMO**

SUMOylation is an essential post-translational modification of proteins that consists in the covalent attachment of the small polypeptide SUMO (Small Ubiquitin-like MOdifier). SUMO was discovered and characterized in the latter half of the 90s. We find SUMO in all the eukaryotic organisms that have been studied, from *Saccharomyces cerevisiae* to *Homo Sapiens*. It was initially discovered in yeast during a genetic screen as a suppressor of mutations in *MIF2* (Mitotic Fidelity of chromosome transmission), which gave it its initial name *Smt3p* (suppressor of Mif-two protein 3) (Meluh and Koshland, 1995). The following year, the human gene *SUMO1* was cloned and named at the time *HSMT3*, which was shown to be involved in various important cellular processes by two-hybrid screening: reparation of DNA via RAD51 and RAD52, component of the PML nuclear bodies (ProMyelocytic Leukemia protein), induction of apoptosis by FAS and TNF (Boddy *et al.*, 1996; Okura *et al.*, 1996; Shen *et al.*, 1996). In 1997, the ATP-dependent and reversible covalent attachment of SMT3 to RanGAP1 (Ran GTPase Activating Protein 1) was demonstrated, and the name SUMO1 was introduced for the first time (Matunis, Coutavas and Blobel, 1996; Mahajan *et al.*, 1997). The sumoylation of RanGAP1 generates its interaction with RanBP2 (Ran Binding Protein 2). RanBP2 has also a SUMO E3 ligase activity. RanGAP1 is strongly and almost constantly sumoylated, therefore making it no surprise that it was the first SUMO substrate that was discovered.

### **Conservation amongst the species**

SUMO proteins are expressed in all eukaryotes. The unicellular eukaryotes, such as yeast or invertebrates like *Caenorhabditis elegans* or *Drosophila melanogaster* have only one gene for SUMO, which is essential for the cellular viability. The superior eukaryotes such as plants and vertebrates express multiple versions of SUMO proteins. The genome of *Arabidopsis thaliana* contains 8 genes that are decoding for the SUMO paralogues (Kurepa *et al.*, 2003). In chicken, xenopus and zebra fish we have two forms of SUMO, SUMO1 and SUMO2, whereas in humans, we have four genes decoding for the SUMO protein, mice only having three. SUMO2 and SUMO3 are 97% identical but share only 50% of sequence identity

with SUMO1 (Hay, 2005). The strong resemblance between SUMO2 and SUMO3 makes it impossible to differentiate them and for this reason they are usually referred as SUMO2/3. Finally, SUMO4 shares 87% of sequence identity with SUMO2 (Owerbach *et al.*, 2005).

In vertebrates, all SUMO isoforms are expressed in all tissues except for SUMO4 who is mainly expressed in the kidney, lymph node and spleen (Guo *et al.*, 2004). However, it is not clear whether SUMO4 is present at the active protein level in-vivo as it cannot be conjugated (Owerbach *et al.*, 2005; Geiss-friedlander and Melchior, 2007; Sinha *et al.*, 2016). Very recently, this protein family grew with the discovery and inclusion of SUMO5, an isoform highly homologous to SUMO1, essential for PML nuclear bodies formation and stability through its conjugation on PML at K160 (Liang *et al.*, 2016).

## **SUMO isoforms**

As indicated previously, although the SUMO peptides share a small sequence identity with ubiquitin, their 3D structures are very close, arranged in a compact globular form, with di-glycine motif at the C-terminus allowing the linkage to the lysine of the substrate.

The structure of the SUMO peptide is distinguished by a N-terminal extension in the opposite direction to the C-terminal end. Most of the sites of SUMOylation in receiving proteins correspond to a motif  $\psi\text{K}\chi\text{E/D}$  where  $\psi$  is a hydrophobic amino acid, K is the modifiable lysine,  $\chi$  is any amino acid and E/D is a glutamine or an aspartate. Functional modification sites were found at the N-terminal end of SUMO2 and 3 indicating the possibility to form SUMO chains, an ability which is not shared with SUMO1. Such chains have been described *in vitro* and *in vivo*. As SUMO1 is deprived of those sites it can only be involved at the end of those poly-SUMO chains or in the mono-SUMOylation (Tatham *et al.*, 2001). This motif of SUMOylation is strongly conserved in yeast, mice and humans (Tang *et al.*, 2008). A proteomic analysis has identified other sites of SUMO2 and 3 linkages (K5 and K7). These linkages appear in a simultaneous manner with with the main linkage in K11, suggesting the possibility for SUMO2 and 3 to form complex compositions in addition to chains (Tammsalu *et al.*, 2014).

Specificity of the SUMO isoforms to the substrates add yet another layer of complexity. Over the past decade, a range of different proteomics studies has reported subsets

of target proteins and sites modified by SUMO proteins. For example, a proteomic study from Vertegaal *et al.* allowed the identification of 53 substrates of SUMOylation. Amongst them, 25 cases present a strong preference for SUMO1, 19 for SUMO2 and 9 were indifferently modified from the one or the other (Vertegaal *et al.*, 2006). Interestingly, the identified substrates were mostly nuclear and included transcriptional regulators (Zfp or Zinc finger protein) and proteins involved in the DNA damage repair. Another proteomic study realized in hyper-expression of the SUMO isoforms identified 232 substrates in the HeLa cells. Likewise, we observe specific substrates of the one or the other isoform, as well as non-specific substrates (Becker *et al.*, 2013). Recently more studies have been investigating the SUMO substrates and a recent compilation of proteomics data has confirmed the existence of at least 1,000 human proteins to be modified by SUMO (Hendriks and Vertegaal, 2016). For the majority of these proteins, the specific SUMO-dependent functions remain unknown, however many fall into functional clusters associated with almost all cellular processes, including the main cancer hallmark<sup>12</sup> functions.

Interestingly and despite this substrate specificity in the conjugation of the isoforms, two different groups have shown that mice without SUMO1 were perfectly viable and underwent normal embryonic development (Evdokimov *et al.*, 2008; Zhang *et al.*, 2008). This indicates that SUMO2/3 is capable to compensate for the loss of SUMO1. To reciprocity was tested recently from another group and their results suggested that SUMO2 is the predominantly expressed SUMO isoform in embryonic stage. Although mouse embryos with inactivated *Sumo3* are viable, mice cannot survive the inactivation of *Sumo2* and die at an embryonic stage, an unexpected finding since SUMO2 and SUMO3 are highly homologous. The study proved that it was not the SUMO2 alone that was indispensable but it is the total level of SUMO2/3 that drives embryogenesis (L. Wang *et al.*, 2014).

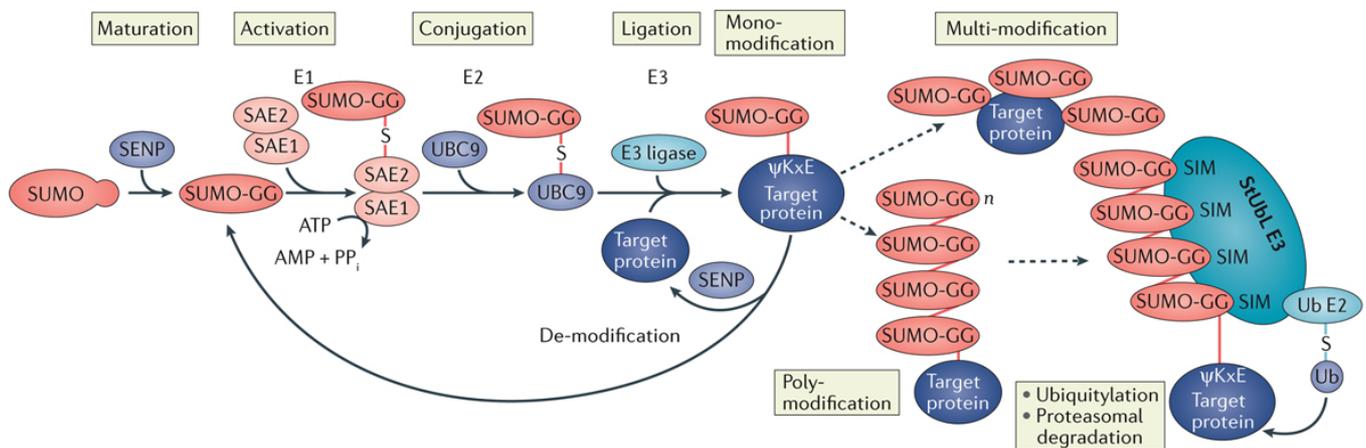
In addition, the dynamic of conjugation seems different amongst the isoforms. On the one hand, SUMO1 appears to be conjugated to its substrates in a relatively stable manner with a small amount of free SUMO1 available. On the other hand, the free SUMO2/3 appears to abandon and are rapidly conjugated to the substrates following stress such as thermal shock (Saitoh and Hinchey, 2000; Seeler and Dejean, 2003).

Finally, the role of SUMO4 as mentioned earlier is controversial: its capacity to be cleaved in a mature form and its capacity to be conjugated, its expression profile in the

organism and its implication in the type I diabetes, constitute of the topics of disagreement between different studies (Guo *et al.*, 2004; Owerbach *et al.*, 2005; Chen *et al.*, 2014). As for SUMO5, although it is highly homologous to SUMO1, it differs from SUMO1 as it is conserved among primate species but appears not to be expressed in mice. In contrast with SUMO1, it is transcriptionally active in selective tissues, with high expression in testes and in blood cells. Regarding its substrates, the study from Liang *et al.*, suggests that SUMO5-conjugated proteins regulate PML and might also regulate the biogenesis of PML-NBs. Moreover, it is possible that SUMO5 plays a role in DNA damage response. Further examination is needed however in order to decipher its roles.

## Enzymatic cascade involved in SUMOylation

The enzymatic process leading to the dynamic and reversible modification of proteins by SUMO is similar to ubiquitination. Consequently, we find the steps of maturation, activation, conjugation, ligation and cleavage (figure 6). However, we observe a strict specificity concerning the enzymes involved in these reactions. The absence of redundancy between the enzymes of the SUMOylation and those of other UBL allows the specific study the former.



**Figure 6 The enzymatic cascade involved in SUMOylation.** The SUMO precursor is processed into its mature form by the action of SUMO-specific proteases (SENPs), which cleave the SUMO carboxyl terminus to reveal a Gly-Gly motif (step 1: maturation). Mature SUMO is activated by and covalently linked to a catalytic Cys in the SUMO-activating enzyme 1 (SAE1)–SAE2 heterodimer (E1 enzyme) (step 2: activation). Activated SUMO is transferred to the catalytic Cys of the SUMO-conjugating enzyme UBC9 (E2 enzyme) (step 3: conjugation). UBC9 catalyses the conjugation of SUMO to Lys residues in target proteins, either independently of or in concert with a SUMO E3 ligase, both of which determine target and Lys specificity (step 4: ligation). SENPs can cleave SUMO from target substrates

(deconjugation), thus releasing an unmodified target and free SUMO (which can re-enter the sumoylation cycle) (step 5: de-modification). Substrates can be mono-sumoylated, multi- and/or poly-sumoylated, the latter with the chain-forming SUMO2 or SUMO3 paralogues. The resultant conjugates can then interact with SUMO-interacting motif (SIM)-containing proteins such as SUMO-targeted ubiquitin ligases (StUbls), which leads to substrate ubiquitylation and subsequent proteasomal degradation (Seeler and Dejean, 2017).

## **Step 1: Maturation**

The synthesis of the new SUMO peptides is caused by immature precursors whose di-Glycine motif at the C-terminal is followed by a sequence of 2 to 11 amino acids (Gareau and Lima, 2010). This sequence has to be cleaved to give rise to the mature SUMO peptides and reveal the di-GLysine motif which is essential for all subsequent steps of the SUMO cycle and mediates the direct linkage of SUMO to each enzyme and target molecule. This maturation occurs thanks to enzymes with endopeptidase activity that are specific for SUMO. Another source of SUMO comes from the recycling of the cleaved from substrates SUMO by enzymes with isopeptidase activity.

In yeast, we encounter two proteases, Ulp1 and Ulp2 (Ubiquitin like protein 1 and 2) that are capable of maturing the Smt3 and to cleave its isopeptide linkage with the substrate. In mammals, three classes of deSUMOylases have been described up to date based on their structure: SENPs (Sentrin Protease, in reference with the old name of SUMO), DES1 and DES2 (Desumoylating Isopeptidase 1 and 2) and USPL1 (Hickey, Wilson and Hochstrasser, 2012).

SENPs, discovered in the early 2000s, are the most described deSUMOylases (Gong *et al.*, 2000). Six SENPs exist: SENP1, -2, -3, -5, -6, -7. Their localization inside the cell appears to partially regulate their substrate specificity. The localization, isoform preference and activity of all the deSUMOylases already mentioned in addition to those mentioned below are summarized in Table 2. They are principally concentrated in well differentiated structures of the nucleus. They can cleave SUMO1 and SUMO2/3, however certain SENPs show a higher affinity for the one or the other isoform. In particular, SENP1 and -2 have specificity for SUMO1 and SUMO2/3 whereas SENP3 and -5 favor SUMO2/3 over SUMO1 (Hickey, Wilson and Hochstrasser, 2012). SENP1, -2, -3 and -5 can deconjugate monoSUMO1 or monoSUMO2/3 and SENP6 and -7 preferentially deconjugate polySUMO2/3 (Alegre and Reverter, 2011) and are the only ones able to edit the SUMO

chains by cleaving the linkages between two SUMO without removing the SUMO which is directly linked to the substrate (Shen *et al.*, 2009; Békés *et al.*, 2011). To date, only SUMO1, -2, -5 have been shown to have a role not only in deconjugation but also in maturation (Kolli *et al.*, 2010).

DESI1 was identified during a screen for partners of the transcriptional repressor BZEL (BTB-ZF protein expressed in effector Lymphocytes). It is located in the nucleus and the cytoplasm and does not share known substrates with SENPs. Its only identified substrate to date is BZEL. DESI1 and DESI2 belong to the PPPDE (Permuted Papain fold Peptidase of Double-stranded RNA viruses and Eukaryotes) class of proteases (Shin *et al.*, 2012).

The third class of deSUMOylases comprises of only one member, USPL1 (Ubiquitin Specific Protease Like 1). It is located in the nuclear structures called Cajal. No substrate has been described up to date, but its role is important for the cellular proliferation, the small nuclear RNA (snRNA) synthesis and the splicing of the pre-messenger RNAs (Schulz *et al.*, 2012; Hutten *et al.*, 2014).

**Table 2 Properties of the SUMO proteases (Hickey *et al.*, 2012).**

Species	Name	Subcellular Localization	SUMO Isoform Preference	Precursor processing	Deconjugation	Chain Editing**
<i>S. cerevisiae</i>	Ulp1	nuclear pore	Smt3	Yes	Yes	No
	Ulp2	nucleoplasm	Smt3	No	Yes	Yes
Mammals	SENP1	nuclear pore and nuclear foci	SUMO1 and SUMO2/3	Yes	Yes	No
	SENP2*	nuclear pore and nuclear foci, cytoplasm	SUMO2/3 > SUMO1	Yes	Yes	No
	SENP3	nucleolus	SUMO2/3	Unknown	Yes	No
	SENP5	nucleolus and mitochondria	SUMO2/3	Yes	Yes	No
	SENP6	nucleoplasm	SUMO2/3	No	Yes	Yes
	SENP7	nucleoplasm	SUMO2/3	No	Yes	Yes
	DeSI-1	cytoplasm and nucleus	SUMO1 and SUMO2/3	Weak	Yes	Yes
	DeSI-2	cytoplasm	Unknown	No	Unknown	Unknown
	USPL1	Cajal bodies	SUMO2/3 > SUMO1	Weak	Yes	Yes

\* SENP2 has at least 3 splice variants that localize differentially in cells. The 589 amino acid version localizes to the nuclear pore.

\*\* Chain editing refers to the ability of a protease to remove SUMO moieties from a SUMO chain without removing the entire SUMO chain from sumo-modified proteins.

## Step 2: Activation

After the maturation the SUMO peptide is activated by the formation of a thioester bond between the glycine of the C terminal and the catalytic cysteine of the E1 enzyme. The E1 enzyme of the SUMOylation is unique. In comparison to the E1 enzyme of ubiquitin which is a monomer, here we have a heterodimer, in which the essential domains for the activation of SUMO are shared between the two parts (AOS-UBA2 in yeast and SAE1-SAE2 in mammals) (Desterro, Rodriguez and Hay, 1998). Focusing on the SAE1-SAE2 (SUMO Activating Enzyme 1 and 2), the one part, SAE1 has an important domain for the ATP-dependant adenylation of the C-terminal Glycine of SUMO, whereas SAE2 had the catalytic cysteine with which SUMO will form the thioester bond of high energy, releasing AMP (Tang *et al.*, 2008).

## Step 3: Conjugation

The next step comprises the transfer of SUMO from the catalytic cysteine of the SAE2 to the catalytic cysteine of the E2 enzyme where a new thioester bond is created, through trans-thioesterification, without ATP consumption. In contrast to ubiquitination, in sumoylation the E2 enzyme is unique and is called UBC9 (UBiquitin Conjugating enzyme 9). As its name indicates, it was first thought to be a new enzyme of the conjugation of ubiquitin involved in the degradation of the B cyclins (Seufert, Futcher and Jentsch, 1995), before its real function was determined. It is localized in different subcellular compartments, in the cytoplasm and the nucleus. It is highly conserved in the eukaryotes with 56% of sequence identity between its orthologs, from *S. cerevisiae* to humans (Kovalenko *et al.*, 1996). Indeed, the Ubc9 of human origin is able to compensate for the loss of UBC9 in yeast (Hateboer *et al.*, 1996). This strong conservation amongst the species underlines the important role of UBC and consequently of SUMOylation in the survival of eukaryotes. Its N-terminal extension permits the binding with SUMO1 and SUMO2/3 in equal affinity (Tatham *et al.*, 2003).

In contrast with the E2 enzymes of ubiquitination, UBC9 is able to interact with certain target proteins and modify them without the intervention of an E3 enzyme. This means that all the necessary information for its sumoylation is integrated in the substrate. The efficiency of the reaction with the target protein however is highly facilitated by the E3 ligases that bring additionally a certain specificity of the substrate.

## Step 4: Ligation

The final step consists of the formation of a covalent isopeptide, the attachment between the C-terminal glycine of SUMO and the receiving lysine of the substrate. As mentioned, they are characterized by their ability to link UBC9 and augment the efficacy of the sumoylation of the substrates *in vitro* and *in vivo*.

Sumoylation differs again from ubiquitination in the fact that the E3 ligases do not form an intermediate covalent attachment with the modifier peptide. Moreover, the E3 of sumoylation are able to auto-sumoylate. Even if significantly less than in ubiquitination, there exist different E3 ligases in sumoylation that can be classified in the separate families detailed below.

### PIAS family:

The proteins of the PIAS family [Protein Inactivator of Activated STAT (Signal Transducer and Activator of Transcription)] were initially named like that due to their ability to inhibit the STAT proteins. These proteins are homologous to the proteins Siz (Siz1 and Siz2) that are the SUMO E3 ligases in *S. cerevisiae* (Johnson and Gupta, 2001; Takahashi *et al.*, 2001). It is the most represented family, and its enzymes have a RING domain, similar to those of certain ubiquitin E3 ligases, which is indispensable for their function. They are able to link the target protein and the UBC9 directly with SUMO through a SIM motif (SUMO interacting motif) that they have. They are encoded by four genes in humans and mice (*Pias1*, -2, -3 and -4, the last one more commonly named *Piasy*) which give rise to different isoforms. The proteins of the family PIAS contain four conserved domains from the one isoform to the other, we notably find a N-terminal SAP (scaffold attachment factor-A/B/acinus/PIAS) motif which allows the link to the DNA, a 'PINIT' motif that along with the RING-type zinc-binding structure contribute to substrate selectivity and, a SIM domain, permitting the interaction with SUMO (Rytinki *et al.*, 2009). What differentiates more the PIAS proteins are their C-terminal domains that we do not find in any other protein and which varies a lot depending on the PIAS protein studied (Figure 7) (Shuai and Liu, 2003).



**Figure 7 Schematic representation of the PIAS structure.** Evolutionarily conserved domains and motifs that mediate nuclear matrix binding (SAP), noncovalent SUMO interaction (SIM), nuclear retention (PINIT), aSUMO ligase activity (Six PIAS [SP] RING) and a C-terminal domain rich in serine and threonine. Note *Piasy*'s C-terminal domain is not rich in serine and threonine (Palvimo, 2007).

In mammals, the compensation of the loss of one PIAS enzyme from the others has not been established. Nevertheless, mice inactivated for *Pias1* are viable and present an augmentation of the production of interferon beta (IFN $\beta$ ) and gamma (IFN $\gamma$ ) making them more resistant to viral infections (Liu *et al.*, 2004). Mice inactivated for *Pias2* present anomalies in the germinal line, however that does not affect their fertility (Santti *et al.*, 2005). Finally, mice inactivated for *Piasy* are viable and phenotypically normal but demonstrate a weak defect in the signaling pathway of IFN $\gamma$  and WNT (Roth *et al.*, 2004; Wong *et al.*, 2004). Our lab has also shown that the cellular senescence of the mouse embryonic fibroblasts (MEFs) inactivated for the *Piasy* was strongly delayed in comparison with control MEFs (Bischof *et al.*, 2006). A recent study revealed a new role of *Piasy* as a positive regulator of intrinsic antiviral immunity to HSV-1 infection uncovering a new role in cellular restriction of DNA viruses (Conn *et al.*, 2016).

Those moderate phenotypes suggest a considerable functional redundancy among mammalian PIAS proteins *in vivo* where none of the inactivations are lethal for the animals and the global sumoylation doesn't seem to be altered. However, up to date, no mice *Pias3*<sup>-/-</sup> has been created and the double *Pias1*<sup>-/-</sup> and *Piasy*<sup>-/-</sup> mutation, causes embryonic mortality (Tahk *et al.*, 2007).

## RanBP2

The second family of SUMO E3 ligases is represented by the protein RanBP2 (Ran Binding Protein 2 or Nup358 for Nucleoporin 358). It is a component of the nuclear pore complex (NPC), located at the cytoplasmic filaments, which is active in nuclear import, but also has an E3 ligase activity. Only few substrates were identified *in vivo*, notably the Topoisomerase II and the Borealin (Dawlaty *et al.*, 2008; Klein *et al.*, 2009). At the nuclear pore a domain within RanBP2 interacts directly with SUMO-modified Ran GTPase-activating

protein 1 (RanGAP1) in a stable complex with the UBC9. Even if it is part of the complex RanGAP1, SUMO1 and UBC9, surprisingly, RanBP2 is not the E3 ligase of RanGAP1. Its presence is required to protect the complex from the deSUMOylases. RanGAP1 can be SUMOylated either by SUMO1 or SUMO2/3 *in vitro*, however *in vivo*, we only encounter the form RanGAP1\*SUMO1. This is due to the fact that SUMO2/3 interacts less with RanBP2 in the context of this complex, which would permit the access to the deSUMOylases (Zhu *et al.*, 2009). This whole complex of RanBP2/RanGAP1\*SUMO1/UBC9 is acting as E3 ligase for the substrates that go through the nuclear pore (Flotho and Werner, 2012).

A recent study revealed another function of RanBP2 in maintaining bile acid (BA) homeostasis through SUMOylation of Small Heterodimer Partner (SHP) at K68, which is required for nuclear transport and the gene repression function of SHP in feedback inhibition of BA biosynthesis that is critical for maintaining BA homeostasis and protecting against liver toxicity (Kim *et al.*, 2016).

## **Pc2**

The polycomb2 protein (Pc2) or CBX4 (Chromobox protein homolog 4) presents also an E3 SUMO ligase activity. It was initially discovered as member of PcG (Polycomb Group protein), forming the important multimeric complexes involved in the histone methylation, hence repressing the transcription, a key epigenetic mechanism for the cellular differentiation. It is structurally different from PIASs and RanBP2 and its localization is restricted in nuclear structures called PcG bodies, generally associated with the pericentromeric heterochromatin (Kagey, Melhuish and Wotton, 2003).

## **NSE2/MMS21**

NSE2 or MMS21 is an E3 sumo ligase which, as the PIAS proteins, have a SP-RING domain. It is a component of a protein complex called SMC5/6 (Structural Maintenance of Chromosomes 5/6) (McDonald *et al.*, 2003; Andrews *et al.*, 2005). Its E3 ligase activity is required for efficient DNA repair and maintenance of genome stability (Potts and Yu, 2005; Zhao and Blobel, 2005; Stephan, Kliszczak and Morrison, 2011; Kliszczak *et al.*, 2012). NSE2 and SMC5 may also be required for sister chromatid cohesion during pro-metaphase and mitotic progression, a role which is apparently independent of SMC6 (Behlke-Steinert *et*

*al.*, 2009). Finally, NSE2 is necessary for normal cell cycle progression in Arabidopsis, as its mutation results in abnormal root development (Huang *et al.*, 2009).

## **The ZNF451 Family**

Recently, another family which is unique in its high specificity for the SUMO2/3 paralogs was discovered: the SNF451 family. Its mechanism is unique, relying on a tandem SIM and its inter-SIM PxRP motif to discharge SUMO-UBC9. There are three different isoforms in this family, ZNF451-1, -2, -3. This family remains largely unexplored. So far, we know that ZNF451-1 modifies PML at established SUMOylation sites (K65/K160/K490) and is involved in its stability. SIM mutations did not result in significant effects indicating that at least some of its functions are independent of its E3 ligase activity (Koidl *et al.*, 2016). Further *in vivo* and *in vitro* studies indicated roles of this family in the SUMO2/3 chain assembly, in stress-induced sumoylation and in the DNA damage response (Eisenhardt *et al.*, 2015).

## **Other E3 ligases**

Amongst the other E3 ligases, we have the HDACs (Histone DeAcetylases), some of which are able to SUMOylate substrates such as MEF2, HIC1 (Hypermethylated In Cancer 1) and PML (ProMyelotic Leukemia protein) (Kirsh *et al.*, 2002; Grégoire and Yang, 2005; Zhao *et al.*, 2005; Yang and Grégoire, 2006; Gao *et al.*, 2008). We also encounter TOPORS (Topoisomerase I binding, arginine/serine-rich, E3 ubiquitin ligase) which has the particularity of presenting an ubiquitin and SUMO ligase activity with substrates such as the tumor suppressor p53 and mSin3A (Pungaliya *et al.*, 2007).

Finally, proteins of the TRIM (TRIPlicate Motif) family have a RING domain with a TRIM/RBCC motif. TRIM proteins and more specifically PML, one of the most SUMOylated proteins inside the cell, have been shown to have an E3 ligase activity permitting the sumoylation of the p53 and MDM2 (Chu and Yang, 2011). The TRIM proteins have been associated with the antiviral response since most of these proteins are induced from type I and II interferons and manifest a role in the antiviral repression via different mechanisms such as ubiquitination (Rajsbaum, García-Sastre and Versteeg, 2014).

## Step 5: DeSUMOylation

An important aspect of SUMOylation is that it is a very dynamic and reversible process. The deSUMOylation is executed by the SUMO proteases that were presented in the chapter “maturation”, the Ulp1 in yeast and SENPs in mammals. These enzymes have an important functional role in the turnover and spatial regulation of SUMO (Mukhopadhyay and Dasso, 2007) and are essential for many cellular processes like chromosome cohesion, mitosis or transcription (Enserink, 2015).

## Regulation of substrate SUMOylation

### Consensus motifs for SUMO conjugation

The conjugation of SUMO to lysine residues constitutes a consensus site. The simplest and most common sequence forms the consensus motif  $\psi K\chi(D/E)$  in which  $\psi$  is an hydrophobic amino acid (leucine, valine, isoleucine or alanine), K is a lysine,  $\chi$  is any amino acid and D/E are either aspartic acid or glutamic acid (Johnson and Blobel, 1999). If this motif is artificially added in a protein it is sufficient to induce SUMOylation *in vitro*, whereas *in vivo* a signal of nuclear importation is also necessary (Rodriguez, Dargemont and Hay, 2001). This consensus motif interacts directly with UBC9, which explains why at least *in vitro*, SUMOylation is possible without the intervention of an E3 ligase (Sampson, Wang and Matunis, 2001). Structures of these complexes between UBC9 and substrates show that consensus motif adopts an extended conformation, in which the lysine fits into a hydrophobic groove on UBC9. This extended conformation that is adopted for productive interactions with the E2 enzyme adds another layer of constraint in the modification by SUMO. Therefore, an important number of sites of SUMOylation are situated in the extended grooves or in non-structured regions of the target proteins (Gareau and Lima, 2010). It is notably the case of SUMO2/3 in which the SUMOylation site is positioned in the N-terminal extension of the peptide.

### Derivatives of the SUMO consensus motif

In addition to the canonical motif, extended motifs have also been described. These include the Phospho-Dependent SUMOylation Motif (PDSM). This motif is encountered in

substrates modified by proline-directed kinases (Yang and Grégoire, 2006) and has the following form  $\psi\chi K(D/E)\chi\chi SP$  where S is a phosphorylatable serine and P a proline. The latter residue of the phosphorylated serine interacts additionally with UBC9 and strongly augments its affinity for the target protein. This mechanism of phospho-dependent SUMOylation is involved in the regulation of the transcription as it is modifying the activity of transcriptional factors, both activators and repressors. It is notably the case of PPAR $\gamma$  (Peroxisome Proliferator-Activated Receptor- $\gamma$ ) or MEF2 (Myocyte Enhancer Factor 2) (Hietakangas *et al.*, 2006).

The increased affinity of UBC9 for the substrates is observed in a similar manner in another extended consensus motif, the negatively charged amino acid-dependent SUMO motif (NDSM). NDSMs comprise negatively charged residues that are C-terminal to the SUMO consensus site in the place of the phosphorylation site of PDSMs, although NDSMs may interact with a different subset of Lys residues on the UBC9 surface (Yang *et al.*, 2006; Mohideen *et al.*, 2009).

A proteomic study of SUMO2 substrates showed that more than half of those are SUMOylated at the level of a lysine situated in the consensus motif. One fourth of other target lysines are in a consensus site slightly modified such as PDSM or even in an inversed consensus site. Only around 10% of substrates are SUMOylated in a very different, comparing to the consensus, site (Matic *et al.*, 2010).

Finally, a third extended SUMOylation motif that was proved to increase the affinity on conjugation of RanGAP1 and ZBTB1 was discovered: the hydrophobic cluster SUMOylation motif (HCSM) (Matic *et al.*, 2010)(figure 8).

### **SIM-dependent SUMOylation**

Another motif other than the lysine consensus that makes SUMOylation possible is the SUMO Interacting Motif (SIM), which permits a non-covalent interaction with the SUMO. To date, the best featured class of SIM consists of a hydrophobic core ((V/I)X(V/I)(V/I)) flanked by acidic amino acids. The increase of the negative charges around the hydrophobic body augments the affinity of the interaction between SUMO and the SIM (Lin *et al.*, 2006; Flotho and Melchior, 2013). The targets of SUMOylation that are SIM dependent can be

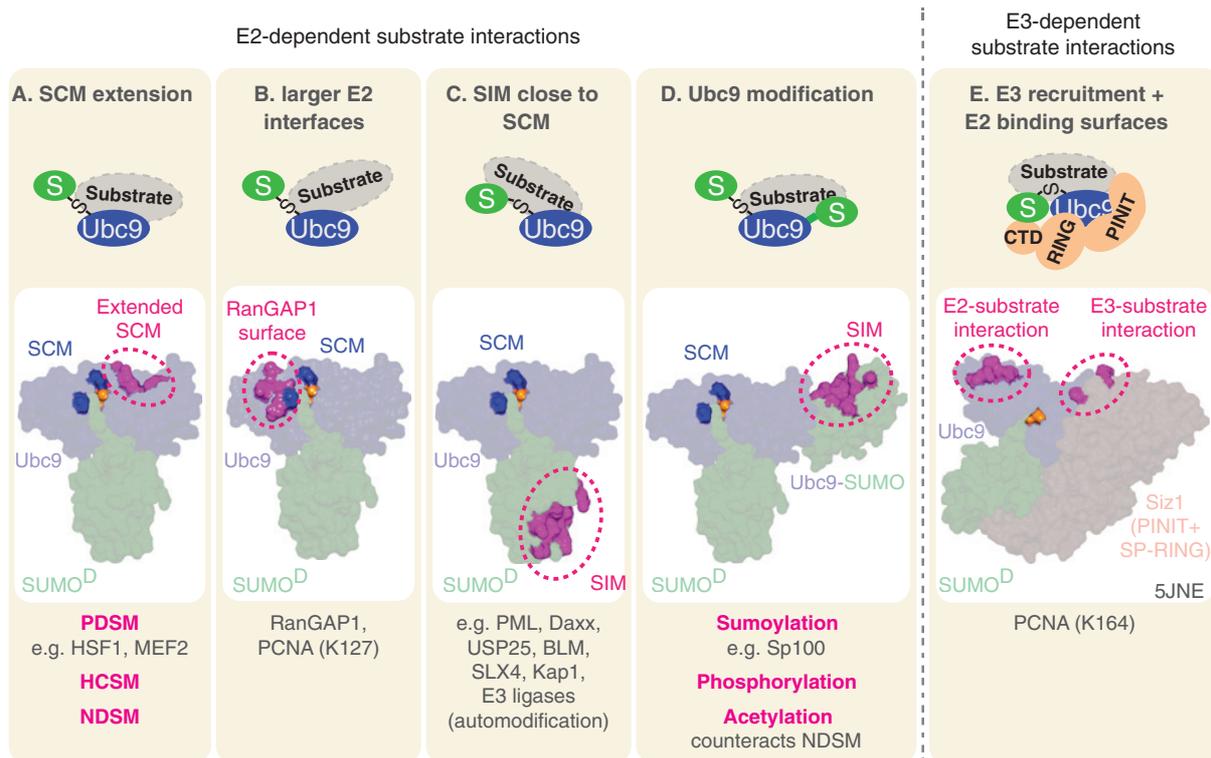
SUMOylated by any lysine and the mutation of one of the lysines doesn't affect the SUMOylation potential of the target protein. As an illustration of such substrates, we have the Daxx proteins (Death domain associated protein), the BLM proteins (Bloom syndrome RecQ helicase-like protein) and the USP25 proteins (Ubiquitin-Specific Protease 25) (Meulmeester *et al.*, 2008; Zhu *et al.*, 2008; Chang *et al.*, 2011). Finally, SIMs are also found in certain enzymes of the SUMO pathway such as UBA2, RanBP2 and the PIAS ligases (figure 8).

### **E3 ligase-dependent SUMOylation**

*In vitro*, SUMOylation is possible in the absence of E3 ligases due to the capacity the UBC9~SUMO complexes have of interacting with numerous substrates. However, in certain cases, the target lysines are inaccessible to the E2 enzyme and an E3 is necessary *in vitro* and *in vivo*. This is the case of the yeast protein PCNA (Proliferating Cell Nuclear Antigen) for example (Pfander *et al.*, 2005) (figure 8).

### **SUMOylation regulated by UBC9 modification**

Another level of complexity in the regulation of substrate interaction is added by different post-translational modifications of UBC9, such as sumoylation, phosphorylation and acetylation of UBC9. Indeed, it has been shown *in vitro* that the affinity to substrates is increased by N-terminal auto-sumoylation of Ubc9 at Lys14 with a SIM close to the consensus motif, which is the case for the sumoylation of the SP100 for example. In particular, sumoylation of Ubc9 enhances the sumoylation of the transcriptional regulator Sp100 while sumoylation of RanGAP1 is impaired. This enhancement is possible thanks to a SIM in Sp100 that creates additional interface for the Ubc9-SUMO binding, different from the typical thioester linkage (Knipscheer *et al.*, 2008). In the case of UBC9 phosphorylation, a similar mechanism was recently suggested, however more analysis needs to be done (Tomasi, Ramani and Ryoo, 2016). Regarding acetylation, UBC9 acetylation at the lysine residue K65 selectively down-regulates the interaction with NDSM-containing SUMO substrates (Hsieh *et al.*, 2013) (figure 8).



**Figure 8 Specificity of interactions determined by the sites of SUMOylation.** The E2 enzyme directly recognizes a SUMO consensus motif (SCM) in a substrate. However, for substrate selection this interaction needs additional binding interfaces such as: A) SCM extensions: PDSM, HCSM and NDSM, B) RanGAP1, C) SIM, D) Posttranslational Ubc9 modifications, like sumoylation, acetylation or phosphorylation or the presence of an E3 E) E3 dependent substrate interactions (Prichler *et al.*, 2017).

## **Complex interplay between SUMOylation and other post-translational modifications**

### **Ubiquitination**

As discussed previously, ubiquitination and sumoylation are both targeting the lysine residues of the target proteins and since their targets are often the same lysines of the same proteins, it is natural to suggest that there is competition between them. The first time that this antagonistic relationship was described was for in the case of the regulator of the NF $\kappa$ B (Nuclear Factor kappa B) pathway. It was shown that the SUMOylation of I $\kappa$ B $\alpha$  (Inhibitor of kappa-b alpha) impairs its ubiquitination. The ubiquitination of I $\kappa$ B $\alpha$  leads to its degradation by the proteasome, permitting the activation of the pathway and the release of the transcription factor NF $\kappa$ B. Therefore, the competition exercised by SUMO to this lysine permits the stabilization of the I $\kappa$ B $\alpha$  and controls the activation of the pathway (Figure 10). However, although ubiquitin and sumo antagonize each other, the direct competition seems to be between phosphorylation and SUMOylation since in order I $\kappa$ B $\alpha$  to be ubiquitinated it needs to be prior phosphorylated and phosphomimic mutant I $\kappa$ B $\alpha$  cannot be SUMOylated (Desterro, Rodriguez and Hay, 1998). Similar relationship between SUMOylation and ubiquitination is observed in another component of the NF $\kappa$ B pathway: NEMO (NF- $\kappa$ B essential modulator). In this case, there is a sequential relationship, where SUMOylation of NEMO facilitates its phosphorylation which in turn is required for the ubiquitination of the same lysine residues of NEMO (Huang and Reichardt, 2003). The relationship between SUMOylation and the NF $\kappa$ B pathway will be detailed later in this manuscript.

Another example where UBC9 and ubiquitin target the same residue but this time with multiple effects for SUMOylation is the PCNA (Proliferating Cell Nuclear Antigen). Competition for the lysine 164 permits the regulation of the DNA repair linked to replication. PCNA is a sliding clamp which encircles the DNA in phase S of the cell cycle and serves as the platform to guide the DNA polymerases and the different factors related to the replication. In response to DNA-damaging agents, PCNA is ubiquitinated. If PCNA is monoubiquitinated in the lysine 164 the replication continues despite the damage of the DNA which may lead to mistakes in replication. This phenomenon is called TLS for translation synthesis. If the TLS fails, PCNA becomes poly-ubiquitinated and a mechanism of homologous recombination intervenes for replication without errors. The SUMOylation of PCNA intervenes during the

replication too. When SUMOylated, PCNA prevents homologous recombination as it recruits Srs2, an anti-recombinogenic helicase that prevents the formation of the recombinase Rad51 (Bergink and Jentsch, 2009).

A proteomic study revealed that 37,8% of the characterized SUMOylation sites were potentially modifiable from ubiquitin too, which suggests that the competition between ubiquitin and SUMO is a common mechanism (Tammsalu *et al.*, 2014).

However, the interaction between SUMOylation and ubiquitination is not limited to targeting of the same lysine residue. Besides the antagonistic or sequential relationships described above, a co-operative relationship between them, in regulating the proteosomal degradation of SUMO target proteins was revealed with the discovery of SUMO-targeted ubiquitin ligases (Geoffroy and Hay, 2009). Another example is the co-regulation of the tumor suppressor p53 where ubiquitination by the RING domain E3 ligase MDM2 recruits the SUMO E3 ligase PIASy, enhancing SUMOylation of p53 at a distinct lysine residue and nuclear export (Carter *et al.*, 2007).

## **Phosphorylation**

SUMOylation is involved in the modification of the phosphorylation machinery and, reciprocally, phosphorylation is involved in the modification of the SUMOylation machinery. In particular, the phosphatase PTP1B (protein tyrosine phosphatase 1B) and kinases such as FAK (focal adhesion kinase), GSK3 $\beta$  (glycogen synthase kinase 3 $\beta$ ), ERK5 (extracellular-signal-regulated kinase 5) and HIPK2 (homeodomain-interacting protein kinase 2) can be modified by SUMO (Kadaré *et al.*, 2003; Roscic *et al.*, 2006; Dadke *et al.*, 2007; Eun Jeoung *et al.*, 2008; Woo *et al.*, 2008). As for the SUMOylation components, SUMO E3s Pc2, RanBP2, TOPORS and PIAS1 can be modified by phosphorylation (Favreau *et al.*, 1996; Roscic *et al.*, 2006; Liu *et al.*, 2007; Park *et al.*, 2008; Stehmeier and Muller, 2009). For instance, in response to DNA damage, HIPK2 is responsible for the phosphorylation of Pc2 which in turn promotes the sumoylation of the HIPK2 (Roscic *et al.*, 2006). As discussed previously, TOPORS is the first E3 enzyme to be present in both ubiquitin and SUMOylation pathways. It has been shown that phosphorylation of TOPORS enhances its activity as ubiquitin E3 ligase without affecting its SUMO E3 activity, suggesting that phosphorylation may regulate the balance of the interaction of the two pathways, ubiquitination and

sumoylation (136). In addition, phosphorylation of UBC9 by CDK1 (cyclin dependent kinase 1) enhances sumoylation by improving the stability and conjugation capacity of UBC9 (Su *et al.*, 2012).

Phosphorylation can have a positive or negative effect in SUMOylation. As was previously mentioned, in the case of PDSM phosphorylation of a substrate can regulate the SUMOylation in a positive manner by reinforcing the interaction between UBC9 and the substrate. A variation of this motif has been described at the ER $\beta$  (estrogen receptor  $\beta$ ), where the last acidic residue is replaced by a serine which impairs the interaction with UBC9. This serine can however be phosphorylated, thereby permitting the interaction with UBC9 and SUMOylation. This motif is called pSuM (phosphorylated Sumoylation Motif) or motif of phosphorylated SUMOylation (Picard *et al.*, 2012).

On the other hand, phosphorylation can sometimes be in competition with SUMOylation. In the case of the protein SATB1 (Special AT rich sequence Binding protein 1), its phosphorylation impairs the interaction and recruitment of the SUMO E3 ligase PIAS1, inhibiting like that its SUMOylation (Tan *et al.*, 2010). Another example is the case of GCM1 (Glial Cell Missing 1) where phosphorylation augments the recruitment of SENP1 and as a consequence its deSUMOylation (Chang, Chang and Chen, 2011).

## **Acetylation**

SUMOylation and acetylation modify in numerous cases the same proteins at the same lysine group, exhibiting competition between them, a similar relationship to that of SUMOylation and ubiquitination. This is the case of the tumor suppressor HIC1 (Hypermethylated In Cancer 1), where the function of HIC1 is controlled by those modifications from a *positive* for the transcription state, when its lysine 314 is acetylated since it decreases the interaction with its co-repressor, to a *negative* when it is SUMOylated since it activates its transcriptional repression (Stankovic-Valentin *et al.*, 2007; Van Rechem *et al.*, 2010). Another example is the transcriptional factor MEF2A which can be both acetylated and sumoylated at the same lysine but the SUMO-acetyl switch is modulated by phosphorylation which favors sumoylation over acetylation (Shalizi *et al.*, 2006).

The complexity of the interaction between SUMOylation and acetylation is particularly reflected in the case of HDACs. HDACs, as mentioned previously, have been reported to function as E3 ligases of SUMO, but have also a function in the removal of acetyl groups from the target proteins which has a repressive effect for the transcription. In addition to this, some of them can also be SUMOylated. As described previously, acetylation of UBC9 has been shown to selectively reduce the SUMOylation of targets with the NDSM motif, which is the case of the transcription factor Elk1. On the other hand, the SUMOylation of the Elk1 is also responsible for the recruitment of an HDAC which, in turn, causes the histone deacetylation of the promoters.

Moreover, the SUMOylation of p53 in the lysine 386 decreases its acetylation. There is no reciprocity to this mechanism since the acetylation of p53 does not affect the SUMOylation (Yang and Sharrocks, 2004). Finally, the acetylation of the SUMO proteins impairs its interaction with SIMs such is the case of the SIMs of Daxx, PML and the PIAS proteins (Cheema *et al.*, 2010; Ullmann *et al.*, 2012).

## **Functional roles of SUMOylation**

### **SUMOylation and stress**

The SUMOylation can be globally regulated in response to cellular stress. Multiple stimuli associated to stress can decrease or increase the global concentration of SUMOylated substrates. Stress shocks such as: heat, osmotic, ethanol and ROS (Reactive Oxygen Species) can increase the SUMOylation mostly by affecting SUMO2/3 conjugation as there is a large pool of free SUMO2/3 in normal conditions and little free SUMO1 (Saitoh and Hinchev, 2000). However, during hypoxia the SUMO1 has been observed to be increased and promoting the hypoxia-specific transcriptional program suggesting that SUMO1 is important for the stress response (Shao *et al.*, 2004). Finally, as a response to different types of stress the stabilization of SUMO4 has been observed, the biological significance of which however is unknown (Wei *et al.*, 2008). Collectively, we observe that compromised SUMOylation often sensitizes cells to stress suggesting that SUMOylation acts like an anti-stress mechanism.

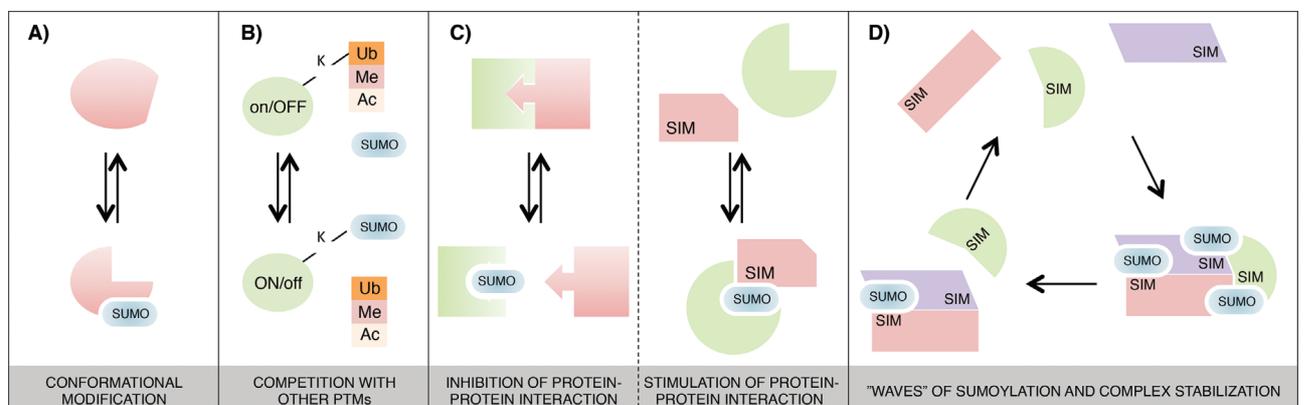
It is important however to mention that specific substrates are differentially modified. After a heat shock an increase of global SUMOylation is observed, but the SUMOylation of some substrates remains unchanged and in other cases decreases. Let us take the example of heat shock and ROS. A proteomic study of hyperSUMOylated substrates during a heat shock shows that a large part of them are involved in stress response (Golebiowski *et al.*, 2009). Different studies have tried to explain the mechanism. One of them suggested that the observed hyperSUMOylation was due to the degradation of SENPs which are thermo-sensitive since that would affect the dynamic balance of conjugation/deconjugation leading to a global hyperSUMOylation (Pinto *et al.*, 2012). This explication does not seem sufficient to explain the enrichment of hyperSUMOylated substrates in response to stress. Another study suggests that following heat shock, SUMO2/3 generated from new protein synthesis was accumulated and that SUMO targets could be unfolded/misfolded proteins produced during the shock (Castoralova *et al.*, 2012). During heat shock, increase in SENP1 likely enhances the cleavage of the SUMO precursor, thus augmenting the maturation and the amount of available active SUMO leading to an increase in the global SUMOylation, all while simultaneously cleaving SUMO selectively from some substrates. During oxidative stress, the treatment of cells with high concentration of H<sub>2</sub>O<sub>2</sub> leads to a massive hyperSUMOylation and whilst it was initially believed that the mechanism was similar to heat shock, it was later shown that high H<sub>2</sub>O<sub>2</sub> concentrations directly inactivate the deSUMOylases. Adversely, in lower concentrations of H<sub>2</sub>O<sub>2</sub>, equivalent of a physiological oxidative stress, a rapid global deSUMOylation is observed. This deSUMOylation is caused by the formation of a reversible disulfide crosslink between the catalytic cysteins of the E1 and E2 enzymes of SUMOylation inhibiting their function without affecting the SENPs (Bossis and Melchior, 2006).

Various studies in yeast, plants and humans have shown that cellular stress could trigger SUMOylation waves where a multisite modification of different repair proteins of a pathway is happening simultaneously. This is notably the case of the response to DNA double strand break (DSB) by homologous recombination (HR) and the response to osmotic stress in yeast (Psakhye and Jentsch, 2012; Lewicki *et al.*, 2015) (figure 9).

## Consequences of SUMOylation in protein conformation or function

SUMOylation can change the stability, the location or influence the activity of the target proteins. There are four different ways that SUMO can affect protein conformation or function, examples of which have already been mentioned previously will be discussed further in this paper. The four mechanisms are the following:

- 1) As described above, SUMO can compete with other PTMs modifying the fate or function of the proteins,
- 2) SUMO can modify the conformation of the target protein,
- 3) SUMO can influence the interaction with other proteins either by a) preventing protein-protein interaction or b) promoting protein interaction through formation of a new binding motif.
- 4) Finally, multiple waves of modification by SUMO are able to simultaneously modify multiple proteins of the same pathway in multiple sites as a response to stress, leading to the stabilization of SIM-containing complexes, enhancing their function (figure 9).



**Figure 9 Consequences of SUMOylation in protein function or conformation** (Chymkowitch, Nguéa P and Enserink, 2015).

## **SUMO and cancer**

The term cancer is not describing a single disease but refers to a range of different diseases that are characterized by an accumulation of genetic mutations. Through these mutations a normal cell is transformed progressively and acquires the characteristics of a cancer cell that are the following:

- 1) A capacity to grow independently of growth signals
- 2) A capacity to evade growth-inhibitory signals
- 3) A capacity to resist apoptosis signals, thus cell death
- 4) Limitless capacity to replicate, thus immortality
- 5) A capacity to induce angiogenesis
- 6) A capacity to infiltrate the tissues and form metastases (Hanahan and Weinberg, 2000)

The cell transformation described above only scratches the surface of the complexity of cancer. Epigenetic factors play important roles in cancer initiation and progression as, for example, complex molecular mechanisms control transcription through chromatin modifications (Berdasco and Esteller, 2013). Moreover, the microenvironment of the cancer cells can contribute in cancer development, an example of which is the inflammatory condition (Karin, 2006; Mantovani *et al.*, 2008). SUMOylation has been attributed to various aspects and types of cancer, both directly and indirectly. For example, amongst the substrates of SUMO we find oncogenic transcriptional factors but also the suppressor of p53 (Kim and Baek, 2006).

Multiple studies have demonstrated the involvement of SUMOylation in many types of cancer. In particular, the deregulation of SUMO machinery has been observed in various types of cancer (table 3).

**Table 3 Deregulation of SUMO enzymatic cascade in different types of human cancer.**  
(Seeler and Dejean, 2017).

Cancer	Enzyme(s)	Expression and observations	Refs
B cell lymphoma*	SUMO2, SUMO3, UBC9, SAE1 and SAE2	Upregulated in high MYC-expressing lymphomas	Hoellein et al., 2004
Bladder	SENP1	Increased <i>SENP1</i> mRNA levels in patient urine; correlated with cancer recurrence	Brems-Eskildsen et al., 2010
	SENP2	Downregulated; associated with enhanced metastatic potential	Tan et al., 2013
Breast*	UBC9	Upregulated in invasive ductal carcinoma and metastases; associated with chemoresistance and poor prognosis	Chen et al., 2011
	UBC9	Upregulated; higher in luminal than non-luminal breast cancer, lower in metastases	Moschos et al., 2010
	PIAS1	Upregulated in early stage breast cancer and invasive ductal carcinoma	Liu et al., 2014
	SENP5	Upregulated; correlated with poor prognosis	Cashman et al., 2014
Colorectal*	SUMO1	Upregulated expression	Zhang et al., 2013
	UBC9	Upregulated in primary cancer	Moschos et al., 2010
	PIAS1	High expression in normal colon and adenoma; downregulated in cancer	Coppola et al., 2009
Gastric*	SAE2	Upregulated; correlated with increased metastatic potential and poor prognosis	Shao et al., 2014
	PIAS1 and PIAS4	Upregulated expression correlated with increased survival in patients receiving second-line docetaxel chemotherapy	Wei et al., 2011
	SENP6	Upregulated; desumoylation of FOXM1 associated with increased FOXM1 stability	Song et al., 2015
	SENP3	Upregulated; associated with lymph node metastasis	Ren et al., 2014
Glioblastoma*	SAE1 and UBC9	Upregulated enzyme and SUMO conjugate levels	Yang et al., 2013 ; Bellail et al., 2014
Glioma*	UBC9	Upregulated in high-grade tumours; inverse relation with expression of miR-214 targeting <i>UBC9</i> mRNA	Zhao et al., 2012
Lung adenocarcinoma*	UBC9	Upregulated	McDoniels-Silvers et al., 2002
	UBC9	Upregulated; lower expression in lymph node metastases	Moschos et al., 2010
	SAE1	Higher transcript levels in lymph node metastases; part of 75-gene transcript metastasis signature	Inamura et al., 2007
Non-small-cell lung	PIAS1	Upregulated in tumours; amplified in cell lines	Rabellino et al., 2012
Small-cell lung*	SAE2	Upregulated in patient samples and cell lines	Liu et al., 2015
Liver*	UBC9	Upregulated	Tomasi et al., 2012
	CBX4	Upregulated; correlated with poor prognosis	Li et al., 2014
Melanoma*	UBC9	Upregulated relative to normal tissue or benign neoplasia (naevi)	Moschos et al., 2007
Multiple myeloma*	UBC9 and PIAS1	Upregulated enzyme and SUMO conjugate levels; associated with poor prognosis	Driscoll et al., 2010
	SENP1	Upregulated; associated with poor prognosis	Xu et al., 2015
Neuroblastoma	SENP1	Upregulated in metastatic disease	Xiang-Ming. et al., 2016
Oral squamous cell carcinoma	SENP3	Upregulated; correlated with tumour differentiation	Sun et al., 2013 ; Ding et al., 2008
Ovarian	PIAS4	Expression level positively correlated with aggressiveness	Sun et al., 2013
Pancreatic	PIAS4	Upregulated	Chien et al., 2013
	SENP1	Upregulated; positively associated with lymph node metastasis and TNM stage	Ma et al., 2014
Prostate*	UBC9	Upregulated; lower at distant metastatic sites	Moschos et al., 2010
	PIAS1	Upregulated transcript and protein levels; required for cell proliferation	Li et al., 2002 ; Hoefler et al., 2012
	SENP1	Enhanced mRNA and protein expression; associated with poor prognosis after radical prostatectomy	Li et al., 2013
Thyroid	SENP1	Transcript upregulated relative to normal thyroid tissue	Jacques et al., 2005
Breast, head and neck, and lung*	UBC9	Upregulated; inversely correlated with miR-30 expression	Wu et al., 2009
Colon, rectum, prostate, ovary and lung	SENP3	Upregulated	Han et al., 2010
Brain, breast, kidney, lung, prostate, skin, liver, oesophagus, stomach and colon	PIAS3	Upregulated in large majority of analysed tumours	Wang and Banerjee, 2004

CBX4, chromobox 4; FOXM1, forkhead box M1; miR, microRNA; PIAS, protein inhibitor of activated STAT; SUMO, small ubiquitin-like modifier; TNM, tumour, node, metastases; UBC9, ubiquitin carrier protein 9; SAE, SUMO1 activating enzyme subunit; SENP, SUMO1/sentrin specific peptidase. \*Cancers with upregulated SUMO, E1 (SAE1/SAE2) or E2 (UBC9) enzymes.

The first time SUMO was associated with cancer was during the discovery of promyelocytic leukemia protein (PML) and the fusion oncoprotein ProMyelocytic Leukemia-Retinoic Acid Receptor  $\alpha$  (PML/ RAR $\alpha$ ) as SUMO substrates (Sternsdorf, Jensen and Will, 1997; Müller, Matunis and Dejean, 1998). Another well established example is the involvement of SUMO in prostate cancer. Let us take a closer look at these two extensively studied examples.

### **Acute Promyelocytic Leukemia (APL)**

PML is a protein that was discovered during studies of APL. APL is a rapid and highly malignant condition which causes sudden hemorrhages. It is a rare condition as it accounts for around 100 new cases per year in France which is around 10% of all APL cases. This leukemia is a consequence of chromosomal translocations giving rise to fusion proteins and, in the majority of the cases, it is caused by a t(15;17) translocation causing the fusion of PML with the receptor of retinoic acid RAR $\alpha$  (de Thé *et al.*, 1990, 1991). The SUMOylation of PML-RAR $\alpha$  is essential for the acquisition of the leukemic phenotype (de Thé *et al.*, 1990, 1991; Wang and Chen, 2008). The PML protein was then largely studied and shown to be involved in numerous cellular processes like the cell cycle, the response to DNA damages, the regulation of transcription, the apoptosis and the senescence (Bernardi and Pandolfi, 2007).

PML is an essential protein for the formation of subnuclear structures called PML nuclear bodies. The number and size of these nuclear bodies varies depending on the cell type, the phase of the cell cycle, stress and nutritional conditions. Their role is crucial for the functions of PML and they are leading to the recruitment of other proteins such as the SP100 (Speckled Protein 100), a transcriptional regulator, and the Daxx, a transcriptional co-repressor involved in apoptosis regulation, both of which are also SUMO substrates (Szostecki *et al.*, 1990; Lallemand-Breitenbach and de Thé, 2010). PML possesses a SIM domain (Shen *et al.*, 2006) and can be sumoylated in three lysines, K65, K160 and K490 (Cheng and Kao, 2012), and its SUMOylation is essential for the maintenance of the integrity of PML nuclear bodies (Shen *et al.*, 2006; Weidtkamp-Peters *et al.*, 2008; Lallemand-Breitenbach and de Thé, 2010). Under arsenic treatment they can be poly-SUMOylated in three more lysines, K380, K400 and K497 (Galisson *et al.*, 2011).

The oncoprotein PML/RAR $\alpha$  is responsible for the non differentiation and the proliferation of the promyelocyte (Brown *et al.*, 1997; Grisolano *et al.*, 1997). The therapy used for the APL consists of the combination of the All Trans-Retinoic Acid (ATRA) and the Arsenic Trioxide (ATO), causing the degradation of the PML/RAR $\alpha$ , constituting the first example of targeted therapy (de Thé and Chen, 2010). The vast majority of patients are now cured with this combined treatment (Dos Santos, Kats and Pandolfi, 2013).

The arsenic treatment causes the polySUMOylation of PML which consequently permits the interaction with the StUbl (SUMO-targeted Ubiquitin Ligase) RNF4 (Ring Finger Protein 4) through its SIM domain. RNF4 causes the polyubiquitination of the PML and the PML/RAR $\alpha$ , leading to their degradation by the proteasome (Lallemand-Breitenbach *et al.*, 2008; Tatham *et al.*, 2008).

## **Prostate cancer**

Prostate cancer is the most common cancer in developed countries. SUMOylation plays critical roles in prostate cancer. Increased SUMOylation and deSUMOylation in prostate cancer cases have been observed to affect the regulation of oncogenes, tumor suppressors and cell cycle. Indeed, SENP1 is highly induced at the majority of the prostate cancer cases (Cheng *et al.*, 2006; Wang *et al.*, 2012). The increase of this deSUMOylase is the result of the increase of *Senp1* by androgens (AR) and the pro-inflammatory cytokines such as interleukine 6 (IL-6). The overexpression of SENP1 results to increase of the transcriptional activity of different factors such as the receptor of androgens, of the oncogene c-Jun and the cycline D1 (Cheng *et al.*, 2004, 2006). A mouse study shows that the overexpression of SENP1, induced by AR, leads to high-grade prostatic intraepithelial neoplasia (HGPIN) by modulating pathways such as the angiogenesis, an important factor of neoplasia development which will be discussed later in the manuscript (Bawa-Khalife *et al.*, 2010).

Other examples, this time of the dysregulation of SUMOylation enzymes, are the overexpression of UBC9 and PIAS1. UBC9 is involved in the transcription of AR target genes and its inactivation causes defects in nuclear organization leading to embryonic lethality (Nacerddine *et al.*, 2005; Kim *et al.*, 2007). However, even if in primary prostate cancer we observe upregulation of UBC9, in metastatic state we have a decrease of UBC9

(Moschos *et al.*, 2010). It has been demonstrated that PIAS1 deficiency leads to small testis with reduced number of sperm cells in mice, which is anticipated since PIAS1 is highly expressed in testis (Santti *et al.*, 2005). PIAS1, which acts as a co-regulator of AR, is highly increased in primary and metastatic tumors. In particular, it has been shown that its inactivation leads to increased transcription of the tumor suppressor p21 and reduced expression of the anti-apoptotic protein Mcl1, thus PIAS1 plays an oncogenic role in prostate cancer (Puhr *et al.*, 2014).

SUMOylation regulates cellular functions of various proteins that play a role in cellular homeostasis with roles in prostate cancer, such as p53, NFκB and PTEN. SUMOylation of p53 increases its transcription and, with the expression of the PIASy, leads to increased induction of senescence that acts as an anticancer mechanism in prostate cancer. The activity of NFκB which is upregulated in prostate cancer, is reduced through its modification by SUMO2 (Kim *et al.*, 2011). PTEN (Phosphatase and TENsin homolog) plays a critical role in prostate cancer as loss of this tumor suppressor leads to the hyper-activation of PI3K (PhosphoInositide 3 Kinase)/AKT/mTOR which is thought to cause prostate cancer (Song, Salmena and Pandolfi, 2012). In prostate cancer samples, overexpression of SENP1 has been observed, and has been shown to promote cell invasion, progression and metastasis (Wang *et al.*, 2012).

## **Regulation of tumor suppressors and oncogenes by SUMO**

It was demonstrated that SUMOylation regulates the functions and the role of PTEN as a tumor suppressor and has been associated with different cancers. As mentioned above, SUMOylation and deSUMOylation of the tumor suppressor PTEN have been associated with the regulation of pathways leading to prostate cancer. Multiple studies have shown the SUMOylation of PTEN in various lysines (K254, K266, K289) leading to an increase of PTEN's activity, which in turn leads to decrease of the PI3K/AKT signaling through increase of its stability (W. Wang *et al.*, 2014) and by favoring its association with the plasmid membrane where it can inhibit the PI3K (González-Santamaría *et al.*, 2012; Huang *et al.*, 2012). Another study demonstrated the SUMOylation of PTEN at a different lysine site (K254) for nuclear localization in contrast with the previous cases. In this case, the SUMOylation had no effect in the PI3K/AKT signaling, but plays a role in the DNA damage repair, not only by promoting its nuclear localization, but also by participating actively in

repair functions (Bassi *et al.*, 2013). Taking all these results into account, we see that PTEN is controlled by both SUMOylation and deSUMOylation, and that based on the lysine site, it contributes to both cytosolic and nuclear localizations of the protein. Finally, PTEN SUMOylation has been shown to compete with ubiquitin and decrease its ubiquitination (W. Wang *et al.*, 2014).

Another example is the case of the complex AP1, a dimer composed of the proto-oncogenes c-Fos and c-Jun (Eferl and Wagner, 2003). AP1 recognizes and binds the 12-Otetradecanoylphorbol 13-acetate (TPA)-responsive elements (TRE), found in many gene promoters (Angel *et al.*, 1987). It has been described that c-Fos is SUMOylated in the lysine 265 and c-Jun in the lysines K257 and/or K229 (Müller *et al.*, 2000; Bossis *et al.*, 2005). It has been shown that SUMOylation reduces the transcriptional activity of the AP1 complex. A recent study has demonstrated a rapid recruitment of the SUMOylated c-Fos to the promoters of the target genes after TPA physiological induction. Surprisingly, in absence of SUMOylation they observed increased TPA transcriptional induction. This suggests that the SUMOylation of c-Fos at the promoters of the target genes plays a buffer role, regulating their expression to optimal levels (Tempe *et al.*, 2014).

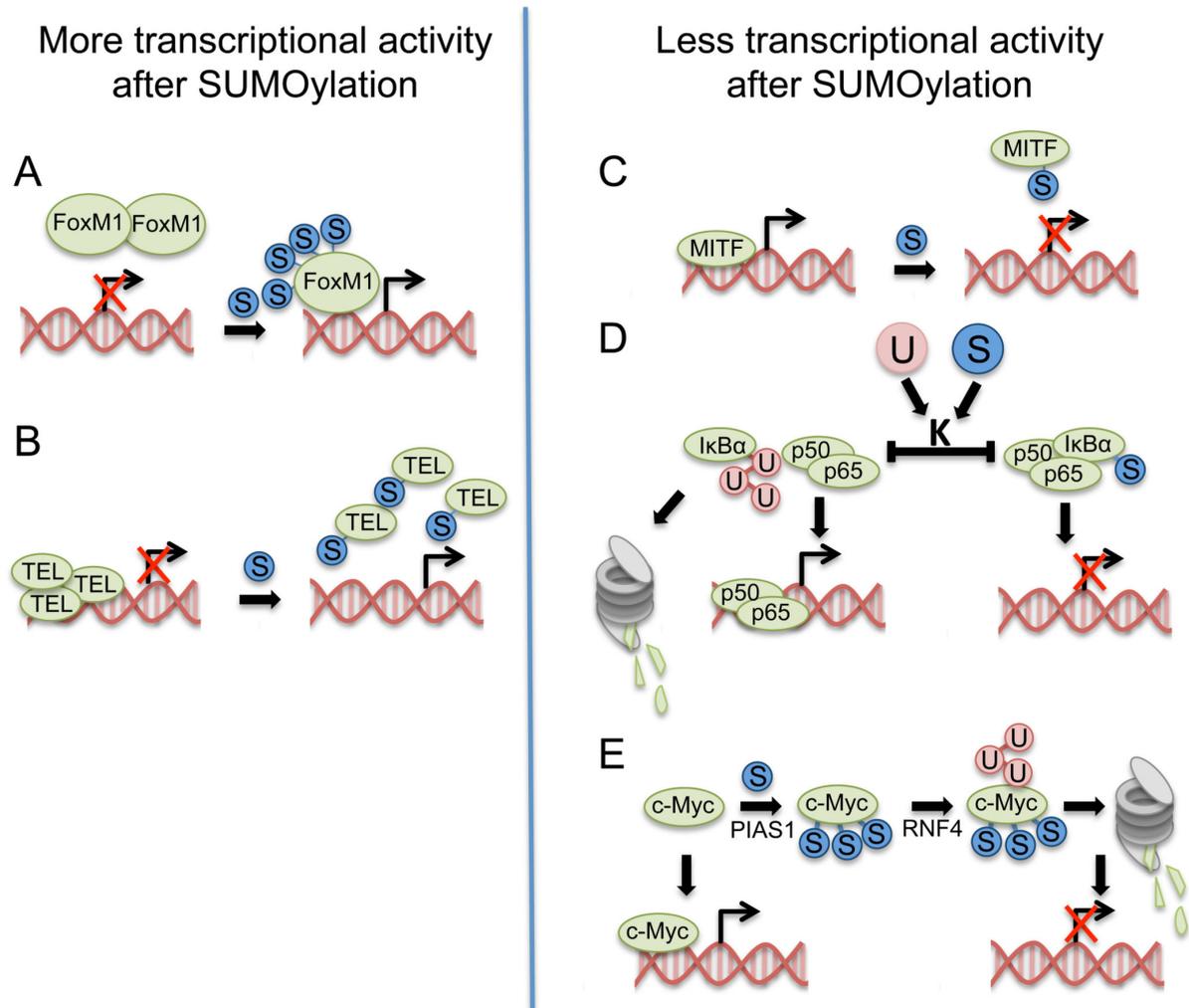
Another oncogene modified by SUMO is the melanoma-lineage-specific microphthalmia-associated transcription factor (MITF). A mutation of this oncogene, called E318K, is responsible for the genetic predisposal of certain patients to melanoma and renal carcinoma. This mutation is situated in the SUMO consensus site and disrupts the SUMOylation, leading to an increase in the levels of other tumor promoting factors such as HIF1, which in turn leads to increase of the transcriptional activity of MITF (Bertolotto *et al.*, 2011; Yokoyama *et al.*, 2011). Taking these results into consideration, we conclude that in this case, SUMOylation plays an onco-protective role preventing tumor initiation and progression as lack of SUMOylation increases the transcription activity of the oncogene (figure 10).

Another oncogene that is SUMOylated is the c-Myc which has been shown to be involved in cell proliferation and apoptosis and has been associated with many different cancers including breast and colon cancer. Recently, c-Myc itself has been shown to be SUMOylated and its SUMOylation leads in turn to its ubiquitination and its rapid degradation by the proteasome (figure 10). The mechanism of post-translational regulation of c-Myc is

unclear and complex as it has been shown that inactivation of PIAS1, leads to a reduction of SUMOylation, which in turn leads to increase of genes induced by c-Myc (González-Prieto *et al.*, 2015), whereas inhibition of SAE2 has the opposite effect (Kessler *et al.*, 2012). In addition, it has been shown that global decrease of SUMOylation can affect cells that have been transformed by the Myc oncogene. In a study it has been observed that decrease of the E1 enzyme SAE1/2 in cells where c-Myc is hyperactivated leads to cell death through disruption of mitosis (Kessler *et al.*, 2012). This phenotype is similar with the results observed in our lab after inactivation of UBC9 in mouse embryos (Nacerddine *et al.*, 2005). Furthermore, inactivation of SAE1/2 in a mouse model of a c-Myc driven breast cancer, resulted to an increase of the survival rate by blocking the cancer progression and metastasis (González-Prieto *et al.*, 2015).

Last but not least, the case of FoxM1 (Forkhead transcription factor 1) which is overexpressed in various cancers such as colon, breast, prostate, lung and liver cancer (Wierstra, 2013) and has been shown to be regulated by SUMO modification (Schimmel *et al.*, 2014) (Figure 10). Like in the case of c-Myc, the role of SUMOylation is unclear with contradictory results in its transcriptional activity. Its transcriptional activity in particular has been inhibited after SUMO1 modification, through increase of ubiquitination and hence degradation or the proteasome and inhibition of mitosis (Myatt *et al.*, 2014). The opposite results were observed when FoxM1 is modified by SUMO2 which leads to increase of transcription and cell proliferation (Schimmel *et al.*, 2014). However, in the first study they used a FoxM1-Ubc9 construct which could be targeted in different lysine site rather than the non-fused FoxM1 of the second study, explaining the different results of SUMOylation.

Several other oncoproteins and tumor suppressors are known to be SUMOylated. Figure 10 depicts some of the mechanisms by which SUMOylation regulates the activity of transcription factors and Table 4 summarizes examples of key tumor suppressors and oncogenes that are modified by SUMO.



**Figure 10 Examples of mechanisms of upregulation or downregulation of the activity of transcription factors by SUMOylation.**

A, B) SUMOylation induces transcriptional activity of FoxM1 and TEL by inhibition of the formation of dimers or polymers respectively. C) SUMOylation of MITF decreases its transcriptional activity whereas a SUMO-deficient mutant increases its transcription. D) As mentioned previously in the manuscript, and will be further discussed below, the ubiquitination of IκBα leads to its degradation by the proteasome, permitting the release of the two subunits p65 and p50 and the activation of the pathway. The competition exercised by SUMO to the same lysine site, permits the stabilization of the IκBα and controls the activation of the pathway. E) SUMOylation of c-Myc via PIAS1 leads to ubiquitylation which in turn leads to decreased transcriptional activity through proteasomal degradation (Eifler and Vertegaal, 2015).

**Table 4 SUMOylation modulates the activity of oncoproteins and tumor suppressors.**

This table shows examples of tumor suppressors and oncoproteins that are modified by SUMO, their canonical function as well as the cancer model they have been observed and the effects of SUMOylation in their activity (Seeler and Dejean, 2017).

SUMO substrate	Canonical function	Cancer (model)	Sumoylation role or mechanism	Refs
<i>Tumour suppressor</i>				
p53	Transcription factor	Various cell lines	Contingent upon monoubiquitylation, sumoylation contributes to nuclear export and attenuation of transcriptional activity	Carter et al., 2007
		Various cell lines	Inhibition of subsequent acetylation and DNA binding, reduced transcriptional activity	Wu and Chiang, 2009
		Various cell lines	Generally mildly enhanced or attenuated transcriptional activity in reporter assays	Stehmeier and Müller, 2009
RB	Transcriptional repressor of S phase genes	Various cell lines	Reduced transcriptional repression by hypophosphorylated form of RB	Ledl et al., 2005
PTEN	PIP <sub>3</sub> phosphatase	Prostate cancer (PC3 cell line mouse xenograft)	Suppression of anchorage-independent and tumour xenograft growth by enhanced PTEN membrane association and downregulation of PI3K–AKT signalling upon abrogation of PTEN sumoylation	Huang et al., 2012
		Colon cancer (HCT116 cell line mouse xenograft)	Essential for nuclear retention of PTEN, critical for DNA repair signalling	Bassi et al., 2013
pVHL	E3 ubiquitin ligase	Renal cell carcinoma (RCC4 and 786-0 cell lines)	pVHL oligomerization via PIAS4-mediated sumoylation, leading to enhanced HIF1 $\alpha$ stabilization, proliferation, migration and clonogenicity	Cai et al., 2010
BRCA1	E3 ubiquitin ligase	Various breast and ovarian cancer cell lines	Enhances ubiquitin ligase activity in DNA double-strand break repair	Morris et al., 2009
<i>Oncoprotein</i>				
MYC	Transcription factor	Osteosarcoma (U2OS cell line)	Poly- or multi-sumoylation-mediated degradation via RNF4 and possibly other StUbls	Gonzalez-Prieto et al., 2015
MDM2	E3 ubiquitin ligase for p53	Colon and prostate cancer (HCT116 and LnCaP cell lines)	SK1 enhances MDM2 sumoylation and activity leading to p53 destabilization	Ding et al., 2012
		Various cell lines	UV-induced desumoylation enhances MDM2 self-ubiquitylation, leading to p53 stabilization	Lee et al., 2006
$\beta$ -Catenin	Transcription factor	Multiple myeloma (RPMI-8226 and NCI-H929 cell lines)	Stabilization of $\beta$ -catenin, enhanced WNT– $\beta$ -catenin signalling, survival and proliferation	Huang et al., 2015
Reptin (also known as RUVBL2)	Transcriptional repressor	Prostate cancer (LnCaP cell line)	Enhanced reptin-mediated transcriptional repression of metastasis inhibitor KAI1 (also known as CD82), leading to enhanced invasive capacity <i>in vitro</i>	Kim et al., 2006
Cyclin D1	G1/S cell cycle regulator	Mouse embryonic fibroblasts	Bypass of RAS oncogene-induced senescence by enhanced nuclear accumulation of SUMO-modified cyclin D1	Wang et al., 2011
<i>Oncogenic fusion protein</i>				
PML–RAR $\alpha$	Nuclear body protein and nuclear receptor transcription factor fusion	APL	Arsenic-induced hypersumoylation and StUbl- (RNF4-) mediated degradation leading to PML nuclear body restoration and apoptosis associated with clinical remission in patients	Tatham et al., 2008 Lallemant-Breitenbach et al., 2008

## SUMO and DNA repair

The malfunction of DNA damage repair mechanisms results in genetic anomalies causing mutations, and those mutations lead to cancers. The genome constantly undergoes damages caused by endogenous mutagens, such as for instance the reactive oxygen species produced during stress, or exogenous causes such as tobacco, alcohol, UV light or natural and synthetic chemical products. This permanent exposure to a risk of mutations requires an efficient and loyal response of the cell to trigger protective mechanisms that will repair the DNA damages (DDR= DNA Damage Repair) (Stratton, Campbell and Andrew F, 2009). SUMOylation is involved in almost all repair mechanisms of DNA damage. The effects of SUMOylation on yeast and mammalian DNA repair proteins is summarized in table 5.

**Table 5 SUMO and DNA repair proteins** (Sarangi and Zhao, 2015).

Substrate	Function(s)	Molecular effect(s) of sumoylation	Modified lysine(s)	E3(s)	Refs
<b>DNA association</b>					
Yku70	DNA end binding	Enhances DNA association	K588, 591, 592, 596, 597	Mms21, Siz1, 2	Hang et al., 2014
Rad1	DNA endonuclease	Lowers DNA binding	K32	Siz1, 2	Sarangi et al., 2014
<b>Protein interactions</b>					
RPA	ssDNA binding	Promotes RAD51 interaction	K449, 577	N/D	Dou et al., 2010
ATRIP	ATR kinase partner	Promotes checkpoint protein interaction	K234, 289	N/D	Wu et al., 2014
Srs2	DNA translocase	Inhibits SUMO-PCNA interaction	K1081, 1089, 1142	Siz1, 2	Kolesar et al., 2012
PCNA	Polymerase clamp	Favors Srs2 interaction; may disfavor Eco1 interaction	K127, 164	Siz1, 2	Pfander et al., 2005 Papouli et al., 2005
Saw1	Nuclease scaffold	Favors Slx4 interaction, disfavors Rad1 interaction	K221	Siz1, 2	Moldovan et al., 2006 Sarangi et al., 2014
<b>Protein level and stability</b>					
FEN1	Flap endonuclease	Promotes its degradation	K168	N/D	Guo et al., 2012
MDC1	DNA checkpoint	Promotes its degradation	K1840	PIAS4	Luo et al., 2012
FANCI	Damage recognition	Promotes its degradation	K4638, 646, 715, 1248, 1288	PIAS1, 4	Gibbs-Seymour et al., 2015
XPC	Damage recognition	Stabilizes the protein	K655	N/D	Wang et al., 2007
<b>Protein solubility</b>					
Sae2	DSB end clipping	Increases soluble Sae2 protein levels	K97	Siz1, 2	Sarangi et al., 2014
<b>Activity</b>					
BRCA1	Ubiquitin E3	Stimulates its E3 activity <i>in vitro</i>	N/D	PIAS1, 4	Morris et al., 2009
<b>Localization and/or recruitment</b>					
XRCC4	DNA ligase scaffold	Promotes nuclear localization	K210	PIAS1, xβ	Yurchenko et al., 2006
TDP1	Phosphodiesterase	Promotes DNA damage site localization	K111	N/D	Hudson et al., 2012
Top2	Topoisomerase	Promotes centromeric localization	K1220, 1246, 1277	Siz1, 2	Takahashi et al., 2006 Bachant et al., 2002 Takahashi et al., 2008
<b>Multiple effects</b>					
Lif1	DNA ligase scaffold	Reduces ssDNA binding and self-association	K301	Siz1, 2	Vigasova et al., 2013
BLM	DNA translocase	Promotes RAD51 interaction, PML localization	K317, 331, 334, 347	N/D	Eladad et al., 2005 Ouyang et al., 2009
Rad52	Recombination mediator	Lowers ssDNA binding/annealing, promotes stability, nucleolar exclusion, Rad51/Ufd1 interaction	K10, 11, 220	Siz2	Sacher et al., 2006 Altmannova et al., 2010 Torres-Rosell et al., 2007
TDG	DNA glycosylase	Decreases DNA binding, promotes intermolecular SIM binding	K330	N/D	Hardeband et al., 2002 Steinacher et al., 2005 Baba et al., 2005, 2016 Smet-Nocca et al., 2011

One of the most studied targets of SUMO in the DNA damage response is PCNA that was previously described in this manuscript as a case of competition with ubiquitin. As mentioned, SUMOylation of PCNA recruits Srs2 which inhibits homologous recombination. Srs2 can also be modified by SUMO interfering with binding of Srs2 to sumoylated PCNA. Adversely, monoubiquitination of PCNA leads to translation synthesis (TLS) despite the damage of the DNA whereas if the TLS fails, PCNA becomes poly-ubiquitinated and a mechanism of homologous recombination intervenes for replication without errors.

Another example of DNA repair pathway in which SUMO plays an important role is the mechanism of repair by base excision (BER= Base Excision Repair). This pathway is activated during an oxidative stress and is responsible for the removal of the damaged base by a DNA glycosylase such as Ntg1 in yeast, NTHL1 and TDG in humans that have been shown to be SUMOylated in response to damage (Hardeland *et al.*, 2002; Swartzlander *et al.*, 2016). For instance, the SUMOylation of TDG (Thymine-DNA-Glycosylase) reduces its binding affinity for DNA *in vitro* by changing its conformation increasing the enzymatic turnover (Steinacher and Schär, 2005; Smet-Nocca *et al.*, 2011). However, the results of a recent *in vivo* experiment suggest that other mechanisms are sufficient to promote the enzymatic turnover of TFG questioning therefore the necessity and role of SUMOylation (McLaughlin *et al.*, 2016).

As mentioned previously, SUMOylation is involved in the DNA double strand break (DSB) response. The E3 SUMO ligases PIAS1 and PIAS4 play an important role in the DSB repair by homologous recombination (HR). Particularly, PIAS-1 and -4 are 1) required for the activity of the E3 ubiquitin ligase BRCA1 at DSBs 2) promote DNA repair through the regulation of the recruitment of DNA repair proteins to DSB and 3) by promoting the SUMOylation of TP53BP1 and BRCA. Studies have shown the accumulation of the SUMO machinery enzymes in a PIAS-1 and -4 dependent-manner, the depletion of which causes decrease in DBS repair (Galanty *et al.*, 2009; Morris *et al.*, 2009).

Finally, SUMO pathway has multiple effects on the ER (Endoplasmatic reticulum) stress response. ER stress results from the accumulation of misfolded proteins. Upon ER stress, in order to re-establish the homeostasis of the cell we have Unfolded Protein Response (UPR) which reduces the unfolded proteins. Upon ER stress the mRNA of the transcription

factor XBP1 is processed by a branch of the UPR, called Ire1, giving rise to the active XBP1s. XBP1s is critical for the ER stress response as it is responsible for the activation of many ER-related genes like those of the ERAD pathway (ER-Associated protein Degradation) and those of protein folding and import (Hetz, 2012). Various studies suggest that SUMOylation has a negative effect on the ER stress response. SUMOylation of XBP1s by PIAS2 as well as interaction with UBC9 has shown to inhibit the transcriptional activity of XBP1s (Chen and Qi, 2010; Uemura *et al.*, 2013). Upon ER stress, deSUMOylation of XBP1s by SENP1 has been shown to boost its transcriptional activity, which is also the case upon depletion of SUMO in *Drosophila* (Jiang *et al.*, 2012; Moore *et al.*, 2013; Lim *et al.*, 2014). It is important to note that at low doses of ER stress, UPR promotes cell viability whereas at higher doses it can induce apoptosis.

The increasing number of DNA repair pathways in which SUMO is involved suggests that a dysregulation of SUMOylation could have a role in the initiation of the oncogenesis process, either by promoting the accumulation of mutations or by impeding the survival of cells with mutations.

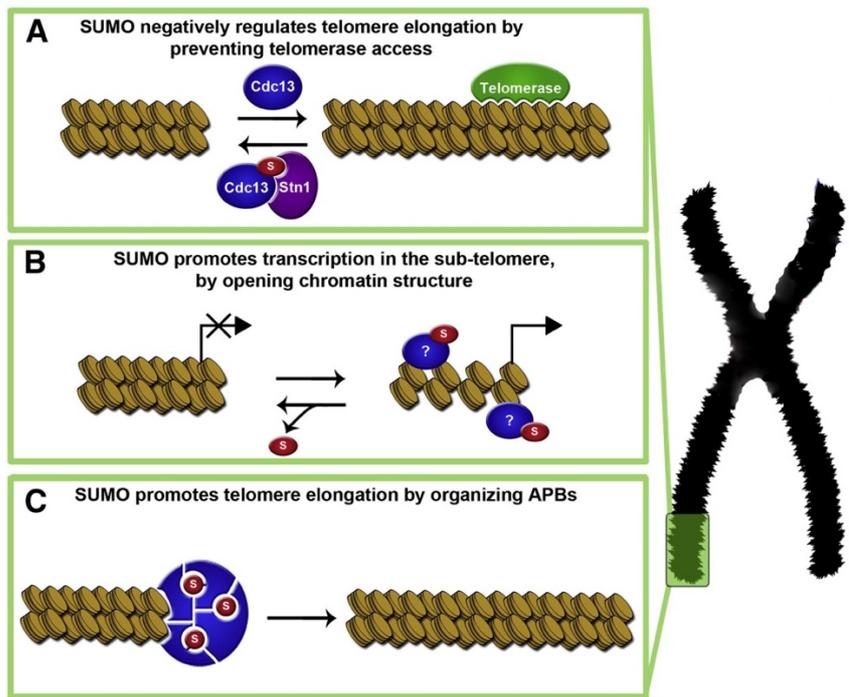
## **SUMO and genome integrity**

Telomeres are repetitive DNA sequences situated at the end of the chromosomes which protect the chromosome ends from being recognized as damaged DNA. In every cell division, telomere length declines and this mechanism contributes to organismal aging. Telomere shortening jeopardizes the integrity of the genome and cells whose telomeres have reached a critical length enter into senescence thus acting as a limiting mechanism of cell proliferation (Bodnar *et al.*, 1998). By limiting the replicative potential of cells, the organism prevents outgrowth of potentially cancerous cells. The majority of cancer cells overcome this potential blockage via overexpression of the telomerase, an enzyme that prolongs the telomeres through addition of DNA repeats (Shay and Bacchetti, 1997). Studies have shown that sumoylation negatively regulates telomere elongation by regulating the activity of a telomere binding protein, Cdc13, which acts as regulator of telomerase recruitment. In particular, SUMOylation promotes the association of Cdc13 with Stn1, a telomerase inhibitor, thereby inhibiting the recruitment of the telomerase (figure 11 A) (Xhemalce *et al.*, 2007). Another study has also demonstrated lengthened telomeres in yeast expressing a SUMO-deficient mutant of Cdc13 (Hang *et al.*, 2011). Moreover, the role of SUMOylation in the

inhibition of the telomerase recruitment is further strengthened by the fact that multiple components that limit telomerase recruitment are known to be SUMOylated (Potts and Yu, 2007; Pebernard *et al.*, 2008; Lu *et al.*, 2009; Ferreira *et al.*, 2011).

Yet another mechanism that can be used by cancer cells is the Alternative Lengthening of Telomeres (ALT) which consists of the homologous recombination of the telomeres and is dependent on assembly of ALT-associated PML nuclear bodies (APBs) (Yeager *et al.*, 1999). Like in the case of PML nuclear bodies, assembly of APBs is regulated by SUMOylation. Studies have shown that knockdown of the E3 ligase Mms21 limits APB formation, thus reduces telomere length and promotes senescence, whereas tethering SUMO or Mms21 to telomeric regions increases APB formation (Figure 11 C) (Chung, Leonhardt and Rippe, 2011).

Furthermore, SUMOylation may regulate telomere elongation by modulating the telomeric heterochromatin. Disruption of the chromatin structure and silence in yeast lead to shortened telomeres suggesting that heterochromatin proteins promote telomere length (Blasco, 2007). Studies have demonstrated that upregulation of SUMOylation promotes chromosome transcription whereas downregulation leads to increased telomeric silencing (Figure 11 B) (Xhemalce *et al.*, 2004; Pasupala *et al.*, 2012). Likewise, a recent study has shown that SUMOylation plays a role in the anchoring of the telomeres to the nuclear pore complex. Telomeres when at critical state, in the absence of telomerase, have been shown to increase SUMOylation of proteins bound to the telomeres which leads to their relocalization to the NPC by the STUbL Slx5-Slx8. This relocalization results to telomere recombination as SUMOylated proteins are removed and that facilitates the telomere DNA repair (Churikov *et al.*, 2016).



**Figure 11 SUMOylation and telomere maintenance** (Cubeñas-Potts and Matunis, 2013).

## SUMO and chromatin

The genome is organized in the form of chromatins consisting of DNA and proteins. This organization permits the regulation of DNA and its packaging into a compact shape. Histones, are the primary category of proteins that constitute the chromatin and are organized in octamers around which the DNA is wrapped, forming the nucleosome. Histones are highly regulated by post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination and SUMOylation (Kouzarides, 2007).

The SUMOylation of histones is conserved from yeasts to mammals. In humans, the SUMOylation of the histone H4 is associated with the repression of transcription through the recruitment of Histone DeAcetylases (HDAC) and of the Heterochromatin Protein 1 gamma (HP1 $\gamma$ ) (Shiio and Eisenman, 2003). In yeast, all four core histones, H2A, H2B, H3 and H4, can be SUMOylated and its SUMOylation is associated with gene repression (Nathan *et al.*, 2006). Even if in most cases SUMOylation is associated with repression of the transcription, a growing number of studies also links SUMO modification to transcriptional activation (Lyst and Stancheva, 2007; Martin *et al.*, 2009).

In humans, ChIP and High-Throughput Sequencing experiments have permitted the collection of information regarding the role of SUMO on chromatin and its association with the regulation of the transcription. Our lab has demonstrated a strong correlation between the regions linked by SUMO and the active promoters (Neyret-Kahn *et al.*, 2013). Another study had previously demonstrated the correlation however the results differ in terms of the function of SUMO at the active promoters (H. W. Liu *et al.*, 2012). On the one hand, the one study shows that decrease of SUMOylation leads to decreased gene expression whereas the results from our lab demonstrate an increase in the expression of the SUMO-targeted genes that encode for histones, ribosomal RNA, transfer RNA and genes involved in protein synthesis. The different methods used for the downregulation of SUMOylation could be a possible explanation for the different outcomes between the two studies. In the case of Liu *et al.*, they observed the effects of the depletion of SUMO1 only in the gene expression, whereas in the case of our lab, Neyret-Kahn *et al.*, targeted the expression of UBC9, reducing like that the global SUMOylation for all the SUMO isoforms.

## **Inflammation**

The inflammation is part of the physiological response in potential harmful conditions for the organism like infection or tissue injury. However, it is well known that chronic inflammation offers to the tumor cells a favorable environment for their proliferation and survival (Mantovani *et al.*, 2008; Grivennikov, Greten and Karin, 2010). Numerous forms of cancer are known for been associated to inflammatory diseases (Mantovani *et al.*, 2008). Moreover, the hyperactivation of key transcriptional factors involved in the inflammation such as NF $\kappa$ B or STATs, has been observed in several cancers. Various studies suggest that SUMOylation regulates the cellular functions of these transcriptional factors suggesting a role of SUMOylation in inflammation and inflammation associated cancers.

As mentioned earlier, upon DNA damage, SUMO1 modifies NEMO, the regulatory subunit of IKK (I $\kappa$ B) having a positive effect in the activation of NF $\kappa$ B pathway (Huang *et al.*, 2003). SUMOylation in this case promotes inflammation and acts in favor of tumor promotion. However, SUMO also targets the inhibitor of NF $\kappa$ B, I $\kappa$ B antagonizing its ubiquitination and consequently blocks its degradation, thus the inhibition of NF $\kappa$ B (Desterro, Rodriguez and Hay, 1998). Therefore, in this case, SUMO acts as tumor suppressor and its

depletion could favor the development of inflammation related tumorigenesis. The PIAS family has been shown to negatively regulate STAT and NF $\kappa$ B. Indeed, the inactivation of PIAS1 leads to an increase of inflammation due to an increase in interferon beta (IFN $\beta$ ) and gamma (IFN $\gamma$ ) through the inactivation of the inhibition by STAT1 (Shuai and Liu, 2005). Even if this increase of inflammation by the inactivation of PIAS1 in mice may initially be beneficial for the resistance against viral infections, the chronic explosion of PIAS1<sup>-/-</sup> mice in inflammatory stimuli could lead to development of auto-immune diseases or chronic inflammation and increase their risk for inflammation associated cancers. Other studies associated the SUMOylation with the repression of inflammation (Pascual *et al.*, 2005; Ghisletti *et al.*, 2010).

Recently, our lab demonstrated that SUMOylation negatively regulates inflammation when mouse macrophages and dendritic cells were genetically inactivated for *Ubc9*. *Ubc9* inactivation resulted in upregulation of basal production of IFN $\beta$  and NF $\kappa$ B dependent inflammatory cytokines such as TNF (tumor necrosis factor), IL-6 (interleukin-6) and CCL4 (C-C motif chemokine 4) (Decque *et al.*, 2016). In this case, decrease in SUMOylation in *Ubc9*<sup>-/-</sup> mice increases the resistance to viral infections and endotoxin shock.

Taking all these examples into consideration we see that SUMOylation can regulate inflammation both positively and negatively. Through the negative regulation we see that SUMOylation could act possible against tumorigenesis by regulating the hyper-immunity that could lead to endotoxic shock, autoimmunity or inflammation related tumorigenesis. On the other hand, as was demonstrated by the example of NEMO, SUMOylation responds to apoptotic stimuli promoting the survival and increasing the inflammation. This will be discussed in detail below. Consequently we observe that, depending on the substrate, SUMOylation can have both positive and negative regulation of the NF $\kappa$ B pathway and the general regulation of innate immunity.

## NFκB

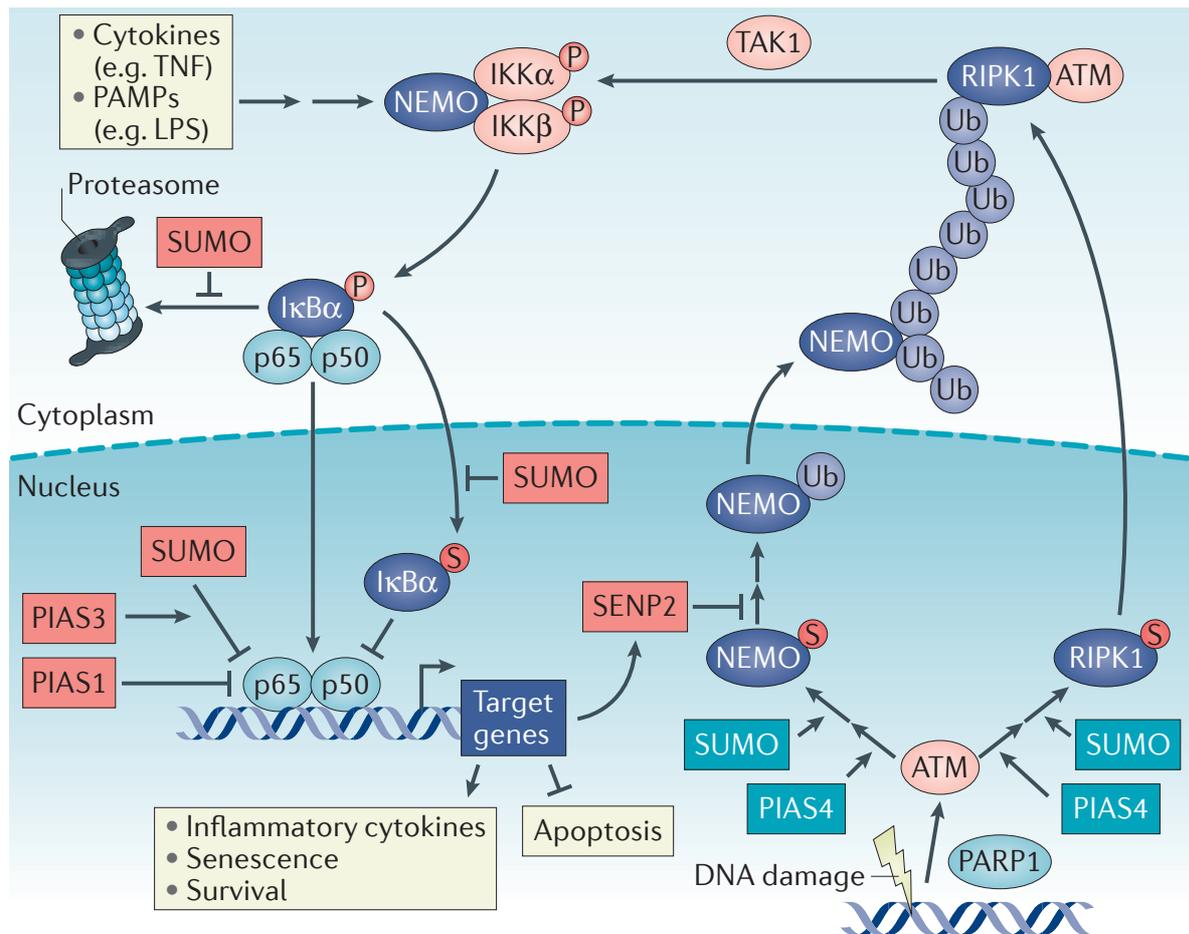
As mentioned earlier, the NFκB pathway apart from promoting inflammation response, has an important role in the regulation of apoptosis stimuli response upon genotoxic stress. Depending on the cell types involved, this regulation can be pro-apoptotic or anti-apoptotic. For instance, in the majority of cancer cases the NFκB pathway is active and regulates anti-apoptotic genes having as a consequence the blockage of the apoptotic processes regulated by caspases. However, even though NFκB has been identified as a pro-survival factor which promotes cancer development, studies have also associated it with increase of apoptosis and senescence sensitivity of tumor cells (F. Liu *et al.*, 2012; Hoesel and Schmid, 2013). Importantly, the canonical NFκB pathway has been shown to promote Fas-mediated apoptosis in tumor cells and particularly colon carcinoma and MEF cells (F. Liu *et al.*, 2012). Upon signal induced activation of the pathway, three different group of genes are activated by NFκB (figure 12) 1) auto-regulatory genes (p50 and p65) 2) immune response genes and activators (cytokines such as IL1, IL2, IL6, IL12, TNFα, IL2Ra) and 3) negative feedback regulators (IκBa).

In its canonical pathway we have the IκB kinase (IKK) phosphorylation which leads to phosphorylation, ubiquitination and degradation of IκB by the proteasome which in turn leads to translocation of the heterodimer p65-p50 from the cytoplasm to the nucleus where it binds the targeted genes and activates their transcription. SUMOylation can compete with ubiquitination preventing the degradation of IκBa, thus acting as a repressor of the pathway. The pathway can be further repressed by PIAS proteins which inhibit the p65-p50 binding to the target genes or by the SUMOylation of p65.

However, in the case of induction of the pathway upon genotoxic stress, SUMOylation has a positive effect on the pathway targeting NEMO, the regulatory subunit of IKK (IκB) acting as an essential mechanism for the activation of the NFκB pathway. In particular, upon genotoxic stress such as DNA damage, we have activation of ATM (Ataxia Telangiectasia Mutated), which leads to SUMOylation of NEMO and RIPK1 by PIAS4 which in turn leads to their ubiquitination and translocation to the cytoplasm (Stilmann *et al.*, 2009). In the cytoplasm, ubiquitinated NEMO and RIPK1 form a complex which is responsible for the recruitment of a kinase that leads to the activation of the IKK by the phosphorylation of its two catalytic subunits, IKKα and IKKβ (Yang *et al.*, 2011). Nevertheless, this pathway is also

inhibited through SENP2 (Lee *et al.*, 2011) or SENP6 (Liu *et al.*, 2013) deSUMOylation of NEMO.

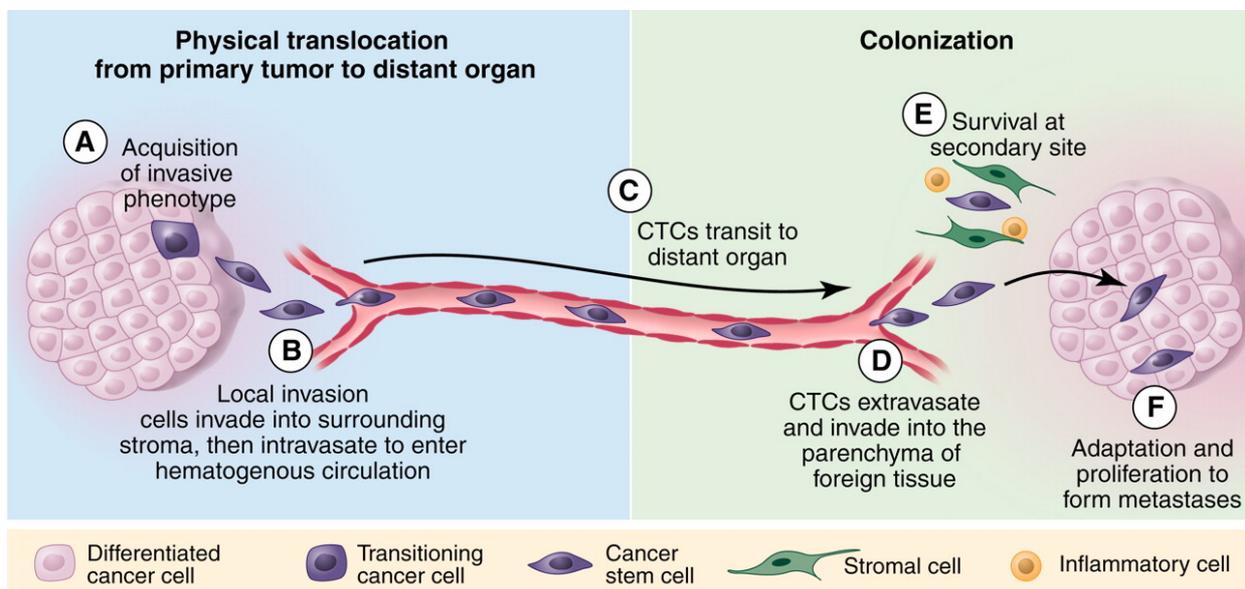
Another pro-survival signaling of NF $\kappa$ B is observed in PML nuclear bodies. Upon DNA damage, the non-canonical I $\kappa$ B kinase IKK $\epsilon$  is SUMOylated and in turn phosphorylates and activates p65 in the PML nuclear bodies.



**Figure 12 Regulation of NF $\kappa$ B pathway by SUMOylation** (Seeler and Dejean, 2017).

## Angiogenesis and metastasis

Tumor cells proliferate rapidly creating large cellular masses. However, in approximately 90% of the cases, cancer-associated mortality is not due to the primary solid tumor but rather to the progress of cancer through metastasis (Chaffer and Weinberg, 2011). Metastasis is the last step of cancer progression, a process containing multiple steps where the cancer cells leave their initial site to colonize other organs or tissues via the circulation of blood (Gupta and Massagué, 2006). The metastatic process includes the migration of cancer cells, the invasion of local tissue, the passage and the transit in the blood circulation, the invasion of foreign tissue and the survival and proliferation in a new environment (Figure 13).



**Figure 13 Representation of the metastasis steps** (Chaffer and Weinberg, 2011).

The impact of SUMOylation in the metastatic process has been associated with the regulation of the TGF $\beta$  pathway and the hypoxia, both of which play important roles in the process of epithelial-to-mesenchymal transition (EMT) of the epithelial tumors.

The contribution of TGF $\beta$  pathway to the tumor development and metastasis has been well established (Leivonen and Kähäri, 2007; Pardali and Moustakas, 2007). The effect of TGF $\beta$  depends on the stage of the tumor. In the initial stages, it inhibits the cellular proliferation and induces apoptosis, therefore acting as a tumor suppressor whereas in advanced stages TGF $\beta$ , which is derived from cancer favors the cellular invasion and metastasis via the induction of the epithelial-to-mesenchymal transition and via angiogenesis.

The linkage of TGF $\beta$  induces the phosphorylation of the cytoplasmic mediators SMAD2 and SMAD3. Phosphorylated SMAD2/3 are linked with SMAD4, translocate in the nucleus and induce the transcription of several TGF $\beta$  target genes. Studies show that one of the receptors of TGF $\beta$ , the TGF $\beta$ R1, is a target of SUMO and its SUMOylation increases its affinity with the SMAD proteins generating a more efficient phosphorylation and an increased transcriptional activity in response to TGF $\beta$  (Kang *et al.*, 2008). Therefore, the SUMOylation has a positive effect in the metastatic process, an assumption that was further confirmed from the reduced metastatic potential of a SUMOylation defective TGF $\beta$ R1 in a mouse model (Lear *et al.*, 2016). SMAD3 and SMAD4 are also targets of SUMO. SUMOylation of SMAD3 is repressive (Stilman *et al.*, 2009) whereas the SMAD4 SUMOylation has contradictory results since it has been shown to enhance its stability and increase the TGF $\beta$  response (Yang *et al.*, 2011) but also to increase the recruitment of the repressor and decrease the TGF $\beta$  response (Chang *et al.*, 2005).

## **Hypoxia**

In order for the tumour cells to undergo metastasis they form vessels that enable them to access the blood circulation, in particular the oxygen in the blood. This capacity is called angiogenesis and is regulated in part by a transcriptional factor HIF1 (Hypoxia-Induced Factor 1). The factor HIF1 which is a part of the response to hypoxia stress in normal and pathological cases of oxygen deprivation, is also used by the tumor cells for their development, as it induces the transcription of multiple genes that promote angiogenesis and metastasis (Brahimi-Horn, Chiche and Pouyssegur, 2007). It has been shown that both SUMOylation and deSUMOylation are involved in the regulation of the hypoxia response. In hypoxia, increased levels of the SUMO machinery have been observed, especially in SUMO1, PIAS4, SENP1 and a SUMOylation enhancer called RSUME (Carbia-Nagashima *et al.*, 2007; Cai *et al.*, 2010; Xu *et al.*, 2010; Sun *et al.*, 2013).

Furthermore, one of the two subunits of the heterodimer HIF1, the HIF1 $\alpha$ , is a target of SUMOylation and its regulation by SUMO suggests an important role of SUMOylation in the control of angiogenesis, with contradictory results of pro- and anti-angiogenesis roles.

On the one hand, two groups have shown that the SUMOylation of HIF1 $\alpha$  increases its stability and its transcriptional activity (Bae *et al.*, 2004; Cheng *et al.*, 2007). In this case,

inhibition of SUMOylation markedly reduces the stability and the activity of HIF1 $\alpha$  inside the tumors, having an anti-tumoral effect by inhibition of the angiogenesis. More studies followed supporting the pro-angiogenesis role of SUMOylation. The protein RSUME increases the global SUMOylation and particularly the SUMOylation of HIF1 $\alpha$ , which leads to its stabilization and augments its transcriptional activity (Carbia-Nagashima *et al.*, 2007). The hyper-expression of SUMO1 leads to an increment of a key factor of angiogenesis called VEGF (Vascular Endothelial Growth Factor, which consequently increases the activation of ERK1/2, MMP13 and the JAK2/STAT5 pathway (Yang *et al.*, 2013). Finally, a group demonstrates that the E3 ligase, Pc2, increases the SUMOylation of HIF1 $\alpha$  in samples of hepatocellular carcinoma, which leads to the upsurge in the production of VEGF in a SUMO dependent manner and is associated with poor prognosis (Li *et al.*, 2014).

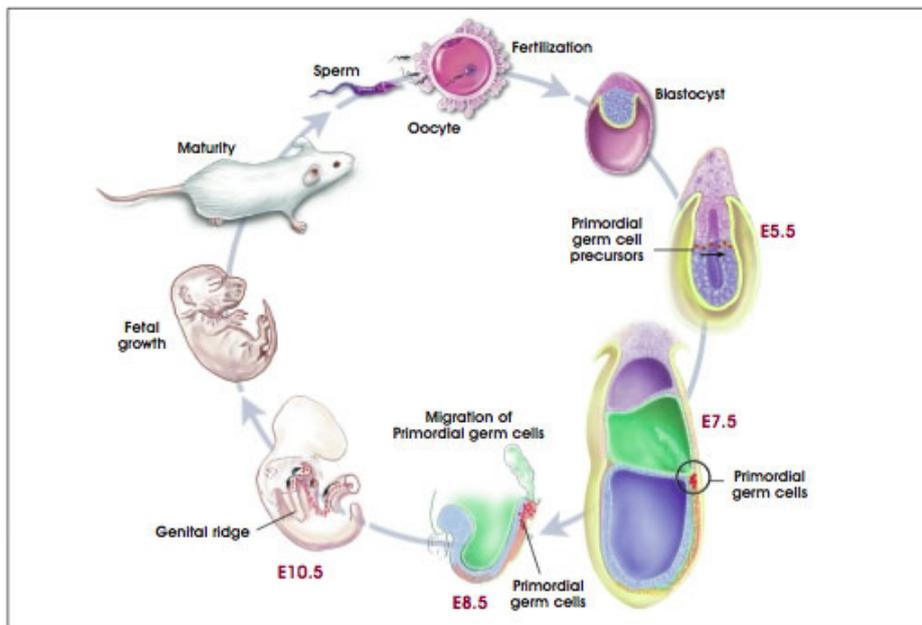
Alternatively, two other groups suggest an opposite role of SUMOylation in anti-angiogenesis. The first group has shown that a mutant HIF1 $\alpha$  which cannot be SUMOylated presents higher transcriptional activity than the wild type HIF1 $\alpha$ , suggesting that SUMOylation decreases the transcriptional activity of HIF1 $\alpha$  (Berta *et al.*, 2007). However, this mutant HIF1 $\alpha$  had other mutations in order to be resistant to siRNA which could affect its general conformation and increase its activity regardless of SUMOylation. The second group, which supports the hypothesis that SUMOylation decreases the transcriptional activity of HIF1 $\alpha$ , has shown that the inactivation of the SENP1 results in a lethal anemia due to a reduced activity of HIF1 $\alpha$  (Cheng *et al.*, 2007). Therefore, this study attributes pro-tumor effect to the deSUMOylation of HIF1 $\alpha$ . Moreover, SUMOylation of the Ub ligase pVHL (Von Hippel-Lindau protein) mediated by PIASy, weakens its ligase activity, which leads to stabilization of HIF1 $\alpha$  (Lin *et al.*, 2003).

Together all these data highlight the complexity of the role of SUMOylation upon hypoxia in a tumor context, as was the case in other stress responses that we've discussed previously. Once again, SUMOylation cannot be considered as a positive or negative regulator of the stress response, thus SUMOylation is indeed an essential pathway for cells with tumor promoting and tumor suppressive properties.

Throughout this manuscript, we have seen that SUMOylation plays important roles in inflammatory, stress and DNA damage responses and impacts signalling of gene networks that regulate anti-apoptotic mechanisms, metastasis and angiogenesis. Furthermore, we have also cited examples in which SUMOylation is involved in cell cycle regulation and telomere maintenance. Undoubtedly, this multitude of SUMO-target substrates involved in almost all biological processes link SUMOylation with multiple steps of tumorigenesis. Therefore, it is of no surprise that different types of cancer are dependent on a functioning SUMOylation system and that, dependent on the substrate, SUMOylation appears to occupy either tumor suppressor or oncogenic functions. Questions remain about whether we could target the SUMOylation system to target cancer and whether the healthy cells will be able to tolerate such inhibitors or enhancers of SUMOylation or deSUMOylation.

## Study of the role of SUMOylation in intestinal and colon cancer

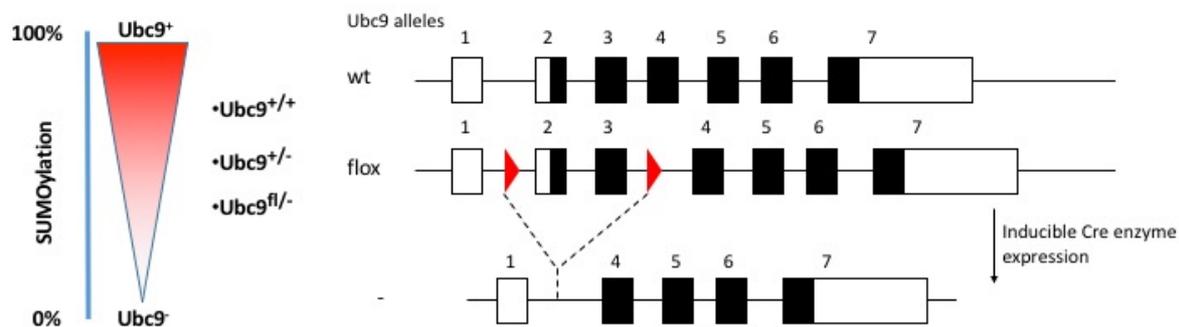
Previous studies in the lab have demonstrated the importance of SUMOylation in the homeostasis of the intestine. In order to study the role of SUMOylation in mice, our laboratory had first chosen to study the loss of function and had constructed a constitutive *Ubc9* knock-out (KO) model. The *Ubc9* was chosen not only because of its pivotal role in the SUMO machinery but also because it is unique and its function cannot be replaced by any other enzyme. This study has shown that the *Ubc9* is essential for the embryonic development and that its absence leads to mortality at the embryonic stage E5.5 (embryonic day 5.5) (figure 14). The phenotypic analysis of the embryonic cells was concentrated on nuclear anomalies and showed that UBC9, and thus SUMOylation, are essential in the embryo for the establishment of the RanGTP gradient, the nuclear morphology, the chromosome condensation and correct segregation. These anomalies led to a massive apoptosis of this mutant embryo (Nacerddine *et al.*, 2005). It is important to highlight however that mice heterozygous for *Ubc9* are viable, fertile and they do not present any detectable phenotype.



**Figure 14 Stages of mouse development.** The main stages are described. The stage E5.5 corresponds to the embryonic day 5.5 in which the embryo is implanted in the uterus. The birth takes place around day E19.5 (Winslow and Kibiuk, 2001).

The embryonic lethality of the *Ubc9*<sup>-/-</sup> mice and the small amount of exploitable material pushed the lab to develop alternative constructs for the study of SUMOylation in

mice. In particular, multiple and unique constructs were generated ranging from conditional knockout and 0% of UBC9 to 100% UBC9 and thus SUMOylation. The role of UBC9 in the intestinal homeostasis was first revealed in the lab using the unique construct of conditional knockout  $Ubc9^{fl/-}/ROSA26-CreERT2$  which consists in the introduction of loxP sites between the exons 1 and 2 and the exons 3 and 4 of the mouse  $Ubc9$  gene. Due to the insertion of the transgene  $CreERT2$  and under the control of the ubiquitous locus ROSA26, the recombination of the sites loxP by the Cre-recombinase can be induced in the presence of 4-hydroxytamoxifen (4OHT). Therefore, the  $Ubc9$  allele created after the 4OHT induction doesn't contain exon 2 nor exon 3 and is not functional (Figure 15). The repetitious injection with 4OHT of mice with this genotype results in their death in six days. A strong decrease of the SYMOylation profile has been observed in all organs, however, the histological analysis reveals a strong phenotype in the intestine. Most notably, the intestinal villi were shortened, the crypt cells were in apoptosis, the LGR5+ cells disappeared and the general villi organization was disrupted. Clearly, the intestine was the principal organ to be affected from the loss of SUMOylation (Demarque *et al.*, 2011).



**Figure 15  $Ubc9$  mouse constructs.** The allele  $Ubc9^f$  consists of two loxP sites flanking exon 2 and 3, permitting their excision and hence the generation of a non-functional allele  $Ubc9$ .

Our mouse models for reduced SUMOylation levels provide a unique opportunity to study the impact of global changes in SUMOylation levels on cancer development. To better understand the mechanisms and functions of the SUMO pathway in both normal and pathologic cell/organism contexts, two approaches have been taken: the characterization of the consequences of the decrease of SUMOylation in the cellular transformation *in vitro* and the analysis of the consequences *in vivo* on different models of colon and intestinal cancer. In an attempt to decipher the mechanism of action, transcriptomic and immunohistochemical analysis were performed and a new mouse model was created and used in order to study the role of the decrease of SUMOylation in stem cells.

# **RESULTS**

## Introduction

A growing body of evidence is demonstrating the implication of SUMOylation in the development of different cancers and notably colorectal cancer. Studies in our lab have demonstrated the crucial role of SUMOylation in the intestinal homeostasis. In this study we are attempting to characterize the consequences of the hypoSUMOylation upon UBC9 targeting in the cellular transformation *in vitro* as well as the colon and intestinal cancer *in vivo*.

In that light, thanks to our unique mouse model for the inducible knockdown of the *Ubc9* gene, we demonstrate that upon knockdown of UBC9, the loss of SUMOylation has a deleterious effect for the transformed cells but not for the primary cells. However, haploinsufficiency of UBC9 accelerates the growth of the transformed cells without affecting the primary cells.

*In vivo*, the heterozygous mice for *Ubc9* have been used to study the colorectal oncogenesis in two complimentary models: a genetic and a chemical model. In the genetic model, the spontaneous intestinal oncogenesis was studied with the use of the inducible model for loss of the Adenomatous Polyposis Coli (*Apc*) gene. In the chemical model, the generation of colon cancer was assessed after the use of the mutagenic agent AzOMethane (AOM) alone and the combination of AOM with the inflammatory agent Dextran Sodium Sulphate (DSS). We see that upon hypoSUMOylation we have an important reduction in the number of polyps and tumor burden in the chemically induced carcinogenesis with AOM-DSS whereas in the genetically induced carcinogenesis we have an important augmentation in the number of polyps and the tumor burden as well as reduced survival expectancy. However, no significant results could be obtained when using AOM alone due to the small number of polyps induced. Immunohistochemical analysis that was performed to characterize the polyps showed difference in terms of number but no difference in terms of morphology of the tumors. Transcriptomic analysis of polyps and normal tissue as well as analysis of the microbiota was performed for both models.

The results obtained *in vivo* suggest an important suppressive and promoting role of SUMOylation in the colorectal carcinogenesis while the results obtained *in vitro* suggest that

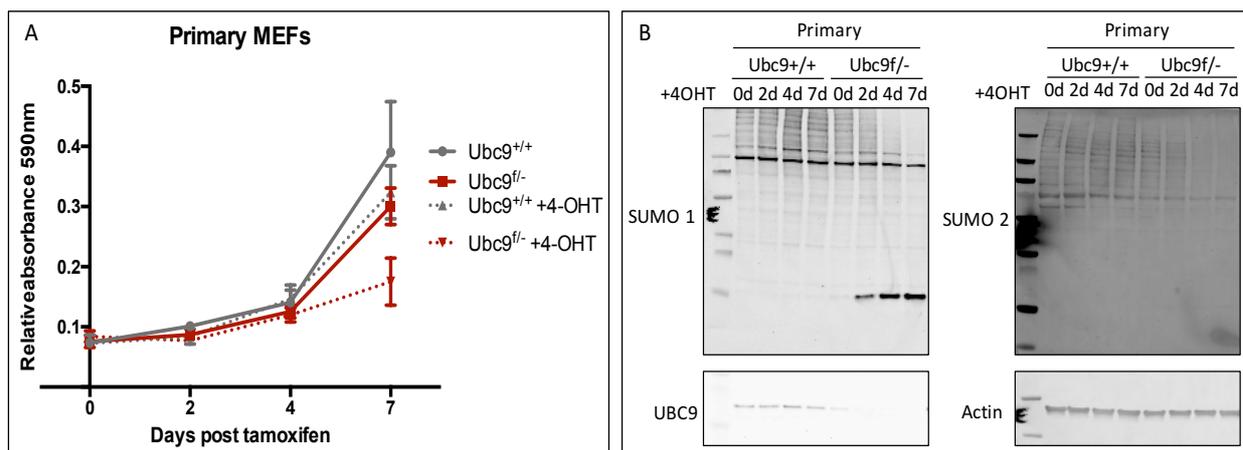
SUMOylation could be a promising potential therapeutic target in the treatment of cancers that has less severe effects on healthy cells.

Additionally, since the cells of origin of colorectal cancer have been shown to be the Lgr5 stem cells, another mouse model was used to study the effect of hypoSUMOylation in stem cells. Interesting initial results suggest that there is a difference in number of stem cells between wildtype and haploinsufficient mice for UBC9.

## SUMOylation and cellular transformation

### Loss of SUMOylation in primary MEFs

As we have discussed above, since SUMOylation is involved in multiple processes that are essential for the cellular homeostasis, naturally our first goal was to determine the consequences of its downregulation in cellular proliferation. To that end, thanks to the collection of *Ubc9* mutants that our lab has generated (described above), *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> mouse embryonic fibroblasts (MEFs) were generated from mice that express the transgene *Rosa26CreERT2*. In the presence of 4-hydroxytamoxifen (4-OHT) the Cre-recombinase is activated leading to the inactivation of the floxed allele (Figure 15). Two different pools of MEFs were used, a pool consisting of a mix of 3 embryos and a pool of 5 embryos generating the same results. The proliferation in short term was analyzed via staining of MEFs with crystal violet fixed in different timepoints, following treatment with 4-OHT or not. Surprisingly, the loss of one allele of *Ubc9*, even if it is reducing in half the quantity of the available protein, doesn't have an impact on the proliferation of the *Ubc9*<sup>fl/-</sup> cells compared to the control *Ubc9*<sup>+/+</sup>. However, the complete loss of UBC9 leads to a strong global deSUMOylation (Figure 16B), and reduces the proliferation of the *Ubc9*<sup>fl/-</sup> cells (Figure 16A). These results indicate that only the complete inhibition of SUMOylation alters the growth of the primary MEFs, whereas the reduction of UBC9 to half does not have an effect on proliferation of the primary cells.

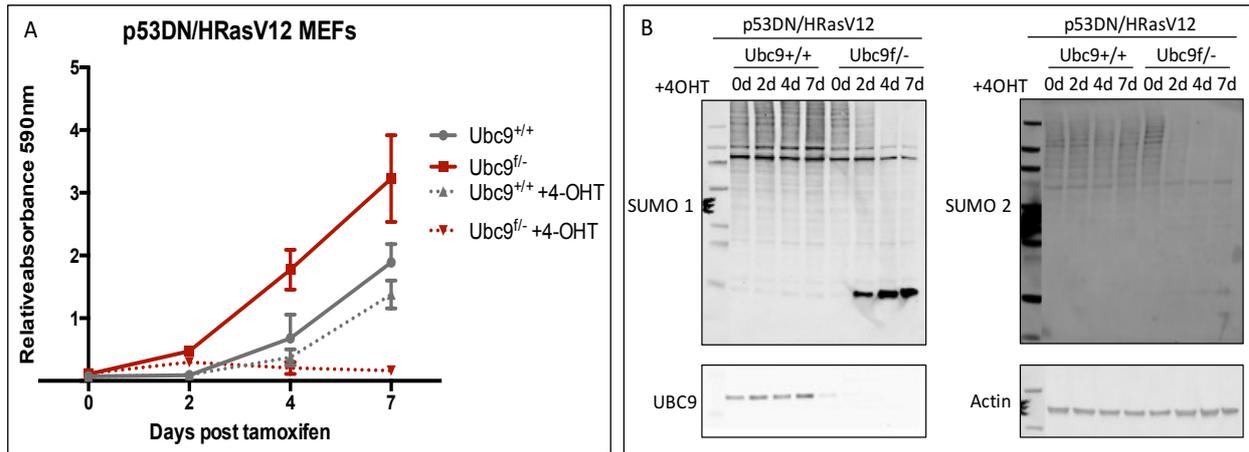


**Figure 16** The inhibition of SUMOylation reduces the proliferation of MEFs. **A:** Growth curve of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> MEFs, upon 0, 2, 4 and 7 days of treatment with 4-OHT or not. Mean  $\pm$  SD, n=3. **B:** Western-blot for SUMO1, SUMO2 and UBC9 in *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> MEFs, upon 0, 2, 4 and 7 days of treatment with 4-OHT or not. Actin was used as loading control. Results shown here represent the pool of 3 embryos. The results of the pool of 5 embryos are presented in the Annex 1.

## **Ubc9 plays important role in the survival and growth of transformed cells**

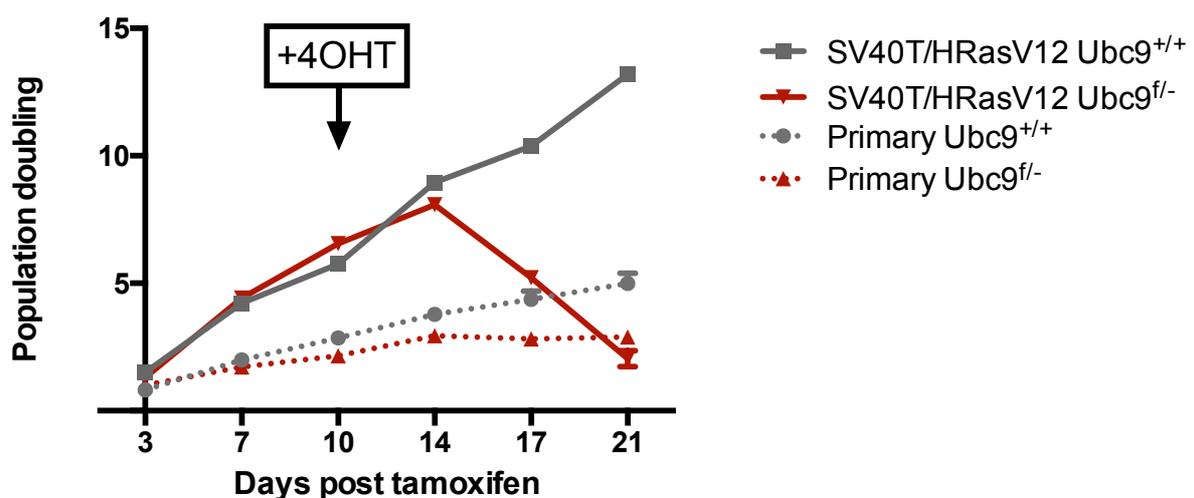
In order to determine the effect of the reduction of Ubc9 in the process of cellular transformation, two different transformed cell lines were used. For the cellular transformation, *Ubc9<sup>+/+</sup>* and *Ubc9<sup>f/-</sup>* MEFs were infected by an immortalization factor and a transformation factor. The one transformed cell line was generated by infecting the MEFs of the pool of 3 embryos with retrovirus coding for dominant negative mutation of the p53 (p53DN) tumor suppressor gene and the constitutively active mutant for the HRAS protein, HRasV12. The other cell line was generated previously in the lab by infecting the pool of 5 embryos with retrovirus coding for the large T antigen of the virus SV40 (SV40T) and the HRasV12. The protein SV40T permits the immortalization of the cells that express it, inhibiting the Rb (Retinoblastoma protein) and the p53 (Tumor Suppressor p53) pathway, two principal components in the control of senescence and apoptosis. The p53DN mutated protein inhibits the p53 pathway. The protein HRasV12 permits the transformation of the cells that express it, as it increases the survival and proliferation signals in a constitutive manner. Mutations as Ras oncogenes are well known to play a role in human cancers with around 30% of human cancers demonstrating altered expression or activation of the Ras oncogenes.

As with the primary cells, the proliferation was analyzed via staining of the transformed MEFs with crystal violet fixed in different timepoints, with and without treatment with 4-OHT. Interestingly, our results demonstrate that the *Ubc9<sup>f/-</sup>* cells proliferate faster than the *Ubc9<sup>+/+</sup>* cells in the absence of 4-OHT suggesting that half dose of UBC9 promotes the proliferation of transformed cells. As in the case of primary MEFs, the complete loss of UBC9 in the transformed MEFs leads to a strong global deSUMOylation (Figure 17B). However, in the case of the transformed cells, our results demonstrate a rapid death with their proliferation reducing drastically already 4 days after 4-OHT treatment suggesting that the transformed cells cannot survive the loss of SUMOylation (Figure 17A).



**Figure 17 Transformed cells cannot survive loss of UBC9 but transformed cells with one allele of *Ubc9* grow faster than the wild-type ones.** **A:** Growth curve of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> transformed MEFs, upon 0, 2, 4 and 7 days of treatment with 4-OHT or not. Mean  $\pm$  SD, n=3. Red: *Ubc9*<sup>+/+</sup>, Grey: *Ubc9*<sup>fl/-</sup>. **B:** Western-blot for SUMO1, SUMO2 and UBC9 in *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> MEFs, upon 0, 2, 4 and 7 days of treatment with 4-OHT or not. Actin was used as loading control. Results shown here represent the p53DN/HRasV12 transformed MEFs derived from the pool of 3 embryos. The results of the SV40T/HRasV12 pool of 5 embryos are presented in the Annex 1.

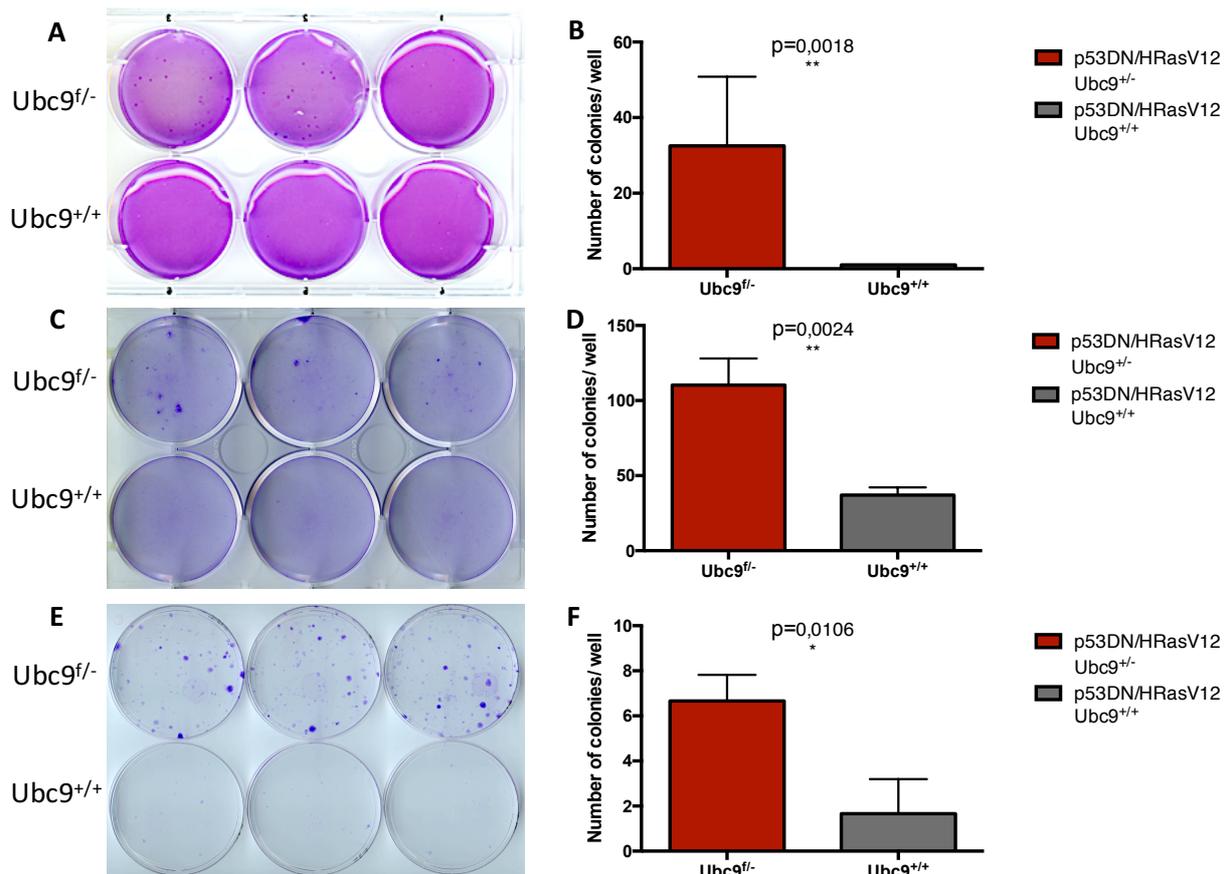
We also investigated the effect of *Ubc9* knockout in primary and transformed MEFs when 4-OHT is introduced later on in a growing population. As in the growth curves described above, we see that transformed cells start reducing and die shortly after inactivation of *Ubc9* whereas the inactivation of *Ubc9* in transformed cells leads to a proliferation arrest but does not kill the cells (Figure 18).



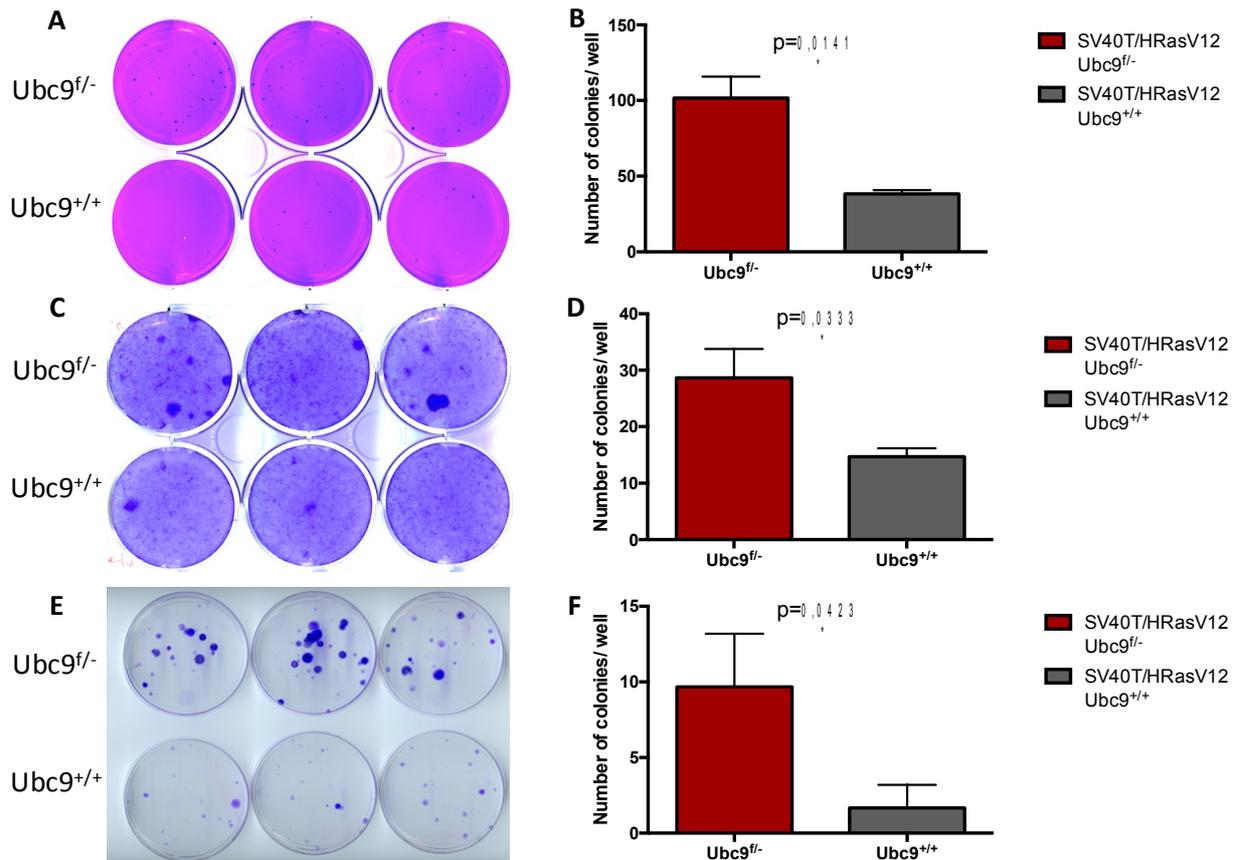
**Figure 18 Transformed cells start dying shortly after 4-OHT treatment.** Growth curve of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> primary and SV40T/HRasV12 transformed MEFs measured with population doubling. The 4-OHT was added after three passages to all cells. Mean  $\pm$  SD, n=2.

## Haplosufficiency of *Ubc9* promotes cellular transformation

For the analysis of the transformed cell characteristics, we performed three assays to address three main characteristics that only transformed cells exhibit in contrast to the primary ones. First we examined the anchorage-independent growth using soft-agar assay. Our results demonstrated that the *Ubc9*<sup>f/-</sup> transformed cells have an enhanced ability to proliferate without attachment to, or spreading onto a substratum comparing to the wild-type *Ubc9*<sup>+/+</sup> (Figure 19A, B, Figure 20A,B). Next, we studied the sensitivity to contact inhibition using focus formation assay. We observed higher number of foci formation of the *Ubc9*<sup>f/-</sup> cells comparing to the wild-type *Ubc9*<sup>+/+</sup> cells (Figure 19C, D, Figure 20C, D). Finally, we studied their ability to grow in low density showing again that the number of foci is higher in *Ubc9*<sup>f/-</sup> cells comparing to the wild-type cells (Figure 19E, F, Figure 20E, F). These data were confirmed in both p53DN/HRasV12 (Figure 19) and SV40T/HRasV12 (Figure 20) transformed cell lines. Together all these data strongly demonstrate that the SUMOylation is involved in the *in vitro* transformation and that the haploinsufficiency of Ubc9 enhances the ability of cells to transform.



**Figure 19 Effects of SUMOylation in cellular transformation.** Soft agar, foci formation and low density assays consistently demonstrated higher capacity of *Ubc9*<sup>f/f</sup> cells to transform comparing to wild-type *Ubc9*<sup>+/+</sup>. **A:** Soft agar assay. Photos of colonies of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>f/f</sup> p53DN/HRasV12 transformed MEFs visualized after 21 days of incubation via crystal violet staining. **B:** Soft agar assay. Comparison of the number of colonies per well between *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>f/f</sup> p53DN/HRasV12 transformed MEFs. Mean ± SD, n≥3. **C:** Foci formation assay. Photos of colonies of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>f/f</sup> p53DN/HRasV12 transformed MEFs visualized after 14 days of incubation via crystal violet staining. **D:** Foci formation assay. Comparison of the number of colonies per well between *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>f/f</sup> p53DN/HRasV12 transformed MEFs. Mean ± SD, n≥3. **E:** Low density assay. Photos of colonies of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>f/f</sup> p53DN/HRasV12 transformed MEFs visualized after 14 days of incubation via crystal violet staining. **F:** Low density assay. Comparison of the number of colonies per well between *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>f/f</sup> p53DN/HRasV12 transformed MEFs. Mean ± SD, n≥3. Unpaired t test with Welch's correction.

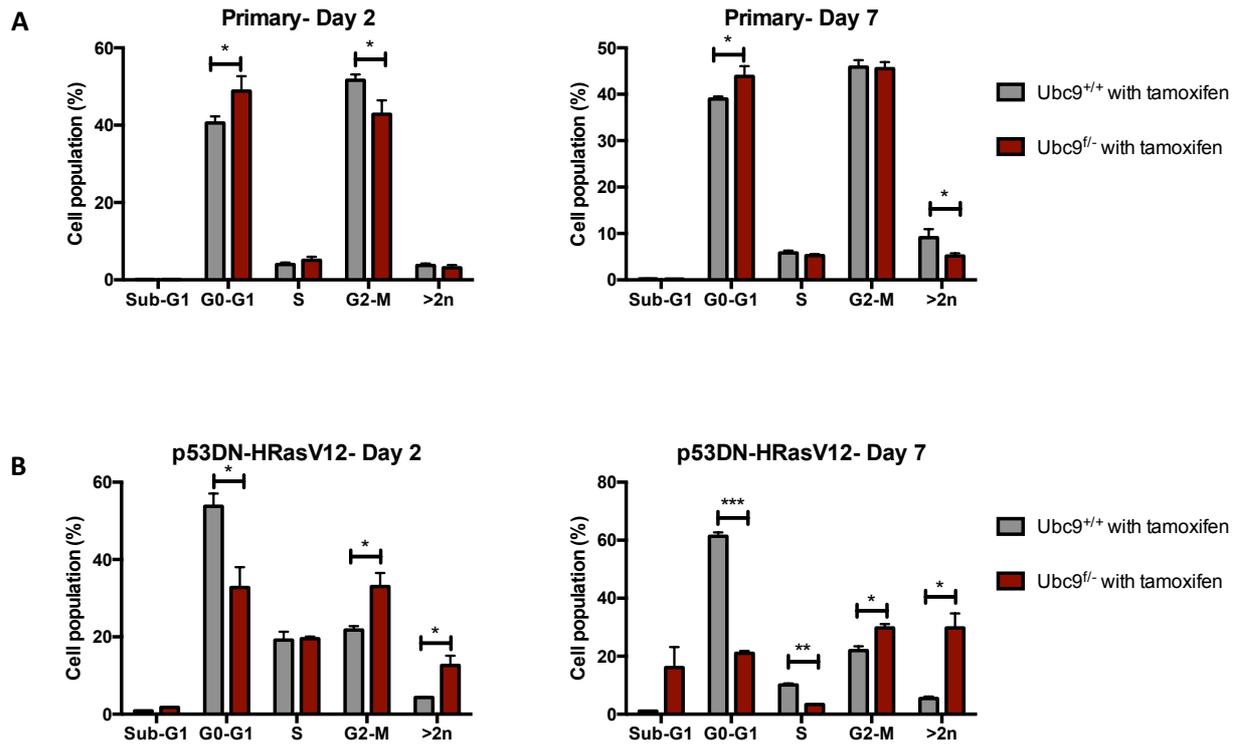


**Figure 20 Effects of SUMOylation in cellular transformation.** Soft agar, foci formation and low density assays consistently demonstrated higher capacity of *Ubc9*<sup>fl/-</sup> cells to transform comparing to wild-type *Ubc9*<sup>+/+</sup>. **A:** Soft agar assay. Photos of colonies of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> SV40T/HRasV12 transformed MEFs visualized after 21 days of incubation via crystal violet staining. **B:** Soft agar assay. Comparison of the number of colonies per well between *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> SV40T/HRasV12 transformed MEFs. Mean ± SD, n≥3. **C:** Foci formation assay. Photos of colonies of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> SV40T/HRasV12 transformed MEFs visualized after 14 days of incubation via crystal violet staining. **D:** Foci formation assay. Comparison of the number of colonies per well between *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> SV40T/HRasV12 transformed MEFs. Mean ± SD, n≥3. **E:** Low density assay. Photos of colonies of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> SV40T/HRasV12 transformed MEFs visualized after 14 days of incubation via crystal violet staining. **F:** Low density assay. Comparison of the number of colonies per well between *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> SV40T/HRasV12 transformed MEFs. Mean ± SD, n≥3. Unpaired t test with Welch's correction.

## The loss of SUMOylation affects the cell cycle

We demonstrated that the knockout of *Ubc9* leads to decrease of proliferation in primary cells and death of transformed cells. In order to determine the effect of the loss of UBC9 in their cellular cycle we performed a cell cycle test after treatment with 4-OHT. In the primary cells, an analysis of the cell cycle does not yield a remarkable accumulation in any specific phase of the cell cycle after 7 days of treatment with 4-OHT (Figure 21A). This means that the growth arrest observed in the growth curves cannot be explained by an arrest in any specific cell cycle phase.

Adversely, in the case of transformed cells, we observed from the 2<sup>nd</sup> day after 4OHT treatment an accumulation of cells in the G2-M phase which remain consistent after 7 days and lead to a decrease of the cell population in G0-G1 phase. These results, together with the observation of a striking percentage of polyploidy after 7 days of 4OHT treatment (~30% accumulate a genome >2n chromosomes) suggest problems in mitosis which are explained by the important role that SUMOylation is known to play in mitosis. Interestingly, we also note an increase in the sub-G1 phase which is an indicator of apoptosis suggesting that the cell death that we observed in the growth curves of transformed cells is due to apoptosis (Figure 21B).

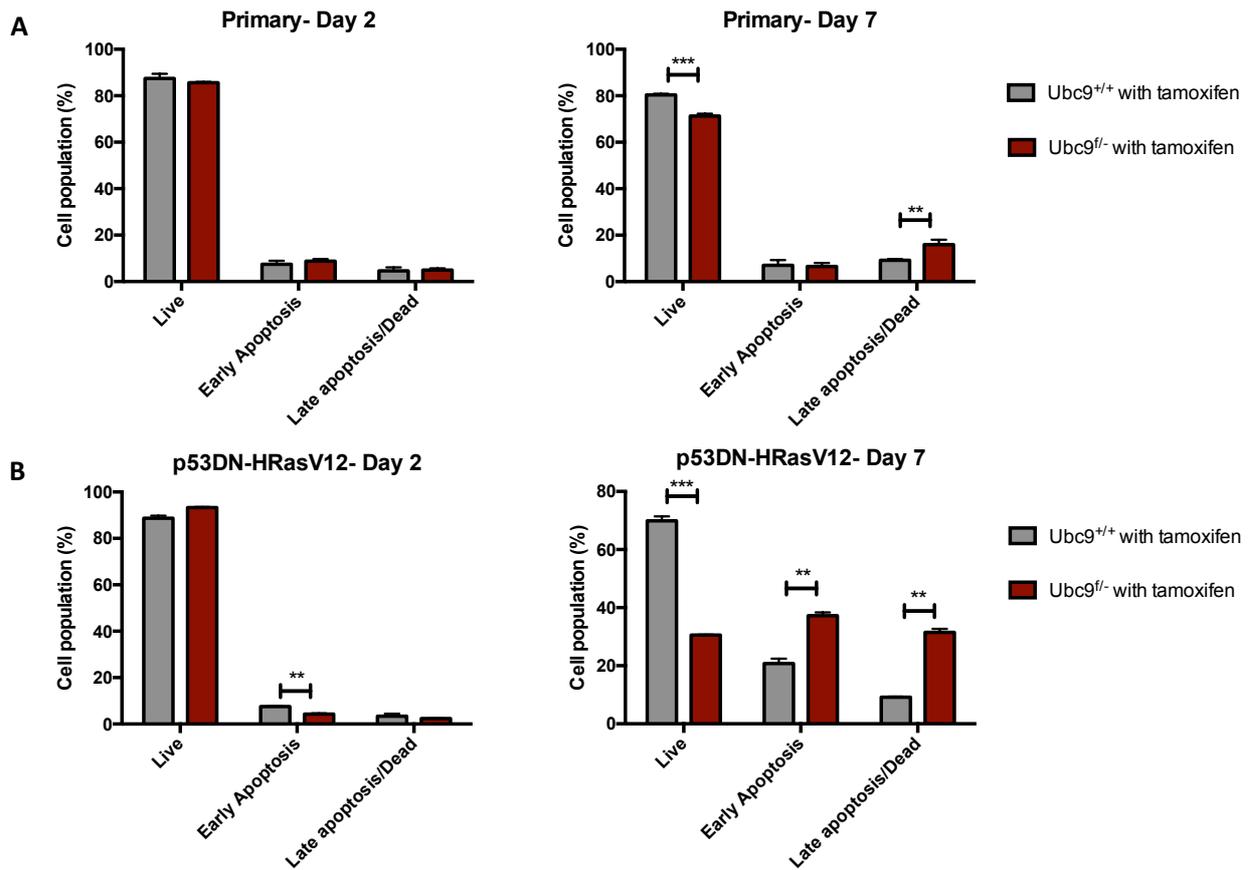


**Figure 21 Effect of loss of SUMOylation in the cell cycle.** **A:** Analysis of the cell cycle of the primary *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> MEFs, after 2 and 7 days of 4-OHT treatment. **B:** Analysis of the cell cycle of the *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> p53DN/HRasV12 transformed MEFs after 2 and 7 days of 4-OHT treatment. Analysis was performed using the Muse Cell Cycle Assay on the Muse Cell Analyzer. Mean  $\pm$  SD,  $n \geq 2$ . p-values:  $p < 0.05^*$ ,  $p < 0.01^{**}$ ,  $p < 0.001^{***}$ . Unpaired student's t-test.

## Loss of SUMOylation promotes apoptosis

As mentioned above, the cell cycle assay demonstrated increase of the sub-G1 phase in the transformed cells suggesting cell death due to apoptosis. To further investigate this result we performed analysis of the apoptosis profile via Annexin V. In the primary cells, only a small amount of cell death is observed with high percentages of viable cells that are not undergoing visible apoptosis both for *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> primary cells (~80 and ~72% respectively) after 7 days of 4-OHT treatment with small increase of late apoptosis/dead cells in the primary *Ubc9*<sup>fl/-</sup> (~16%) comparing to the *Ubc9*<sup>+/+</sup> (~9,2%) (Figure 22A).

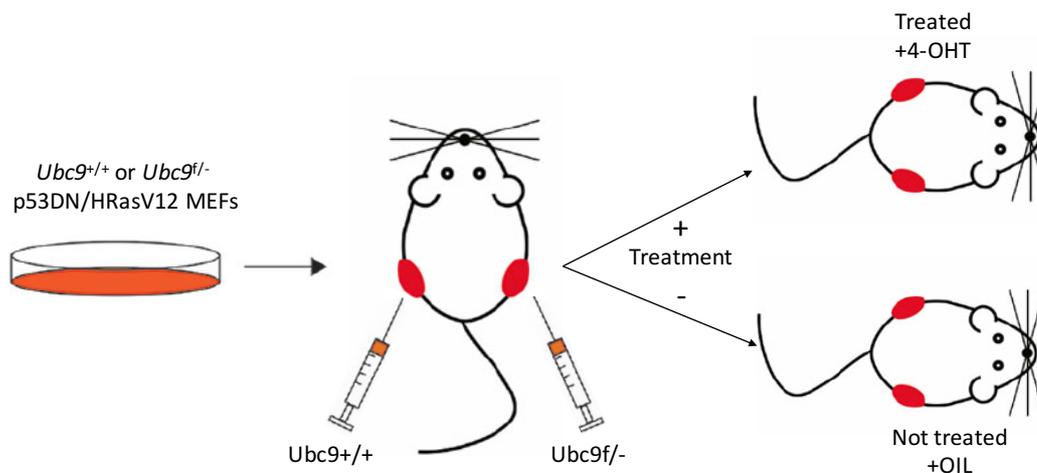
On the contrary, we see a very low amount of *Ubc9*<sup>fl/-</sup> transformed cells that do not undergo visible apoptosis (~30%) comparing to the *Ubc9*<sup>+/+</sup> (~70%) after 7 days of 4-OHT treatment (Figure 22B). Furthermore, we observe an increase in the apoptosis of *Ubc9*<sup>fl/-</sup> transformed cells comparing to *Ubc9*<sup>+/+</sup> in both early apoptosis (~37% and ~20% respectively) and late apoptosis (~32% and ~9% respectively) (Figure 22B). It is clear that the transformed cells cannot survive the knockout of *Ubc9* which causes their death by apoptosis.



**Figure 22 Loss of SUMOylation promotes cellular death of the transformed cells via apoptosis.** **A:** Analysis of live cells, early and late apoptosis and cell death of the primary *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>-/-</sup> MEFs, after 2 and 7 days of 4-OHT treatment. **B:** Analysis of live cells, early and late apoptosis and cell death of the *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>-/-</sup> p53DN/HRasV12 transformed MEFs after 2 and 7 days of 4-OHT treatment. Analysis was performed using the Muse Annexin V & Dead Cell Assay on the Muse Cell Analyzer. Mean  $\pm$  SD,  $n \geq 2$ . p-values:  $p < 0.05^*$ ,  $p < 0.01^{**}$ ,  $p < 0.001^{***}$ . Unpaired student's t-test.

## Tumor xenografts

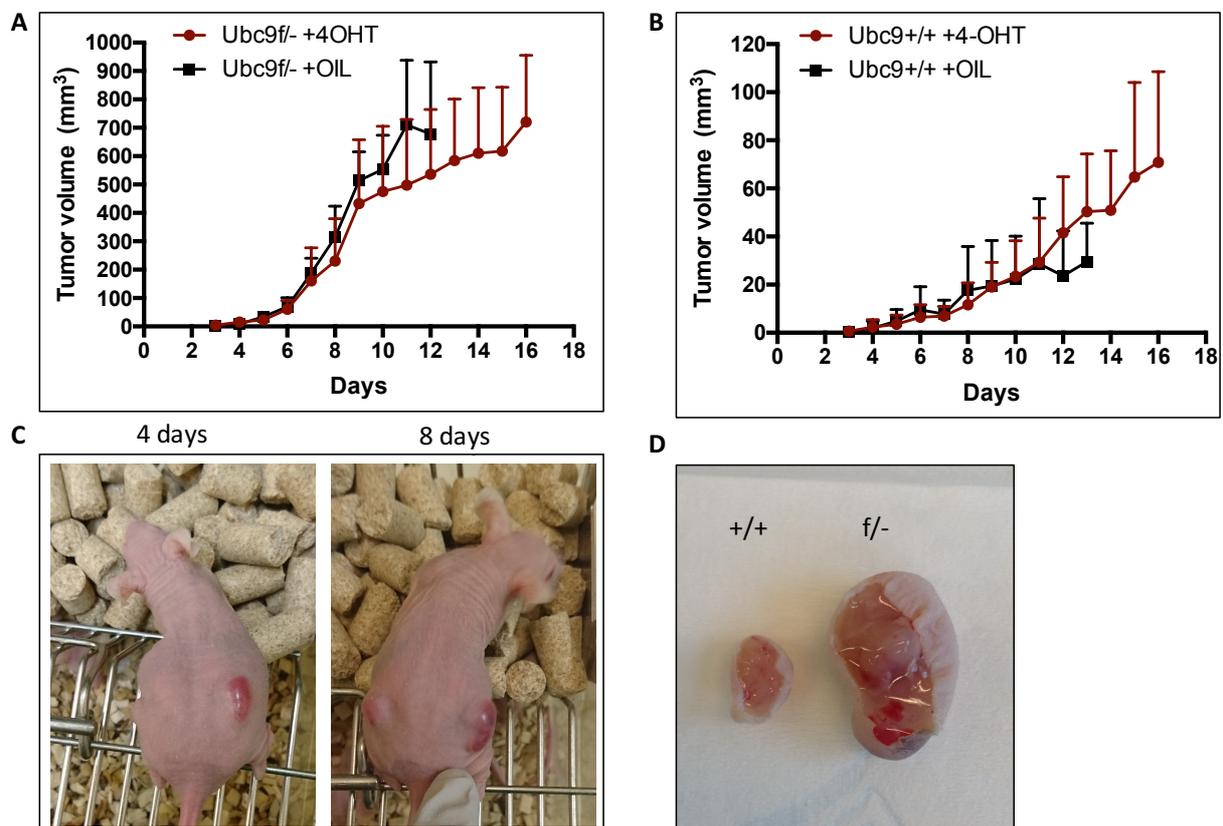
The establishment of tumor xenografts, is a widely used method that consists of one injection of a high number of transformed cells subcutaneously to immunodeficient mice. These mice allow the *in vitro* established cell lines to be propagated subcutaneously, leading to the formation of solid tumors that continue to grow every day. In order to evaluate the transformation capacity *in vivo*, we performed xenograft test in nude athymic mice using one subcutaneous injection of 1 million  $Ubc9^{+/+}$  and  $Ubc9^{fl-}$  p53DN/HRasV12 transformed MEFs with simultaneous 4-OHT treatment or not (Figure 23).



**Figure 23 Schematic representation of the establishment of tumor xenografts from  $Ubc9^{+/+}$  and  $Ubc9^{fl-}$  p53DN/HRasV12 transformed MEFs.** Nude athymic mice were injected subcutaneously with  $Ubc9^{+/+}$  MEFs in the left flank and with  $Ubc9^{fl-}$  MEFs in the right flank and once the tumors appeared mice were treated or not with 4-OHT.

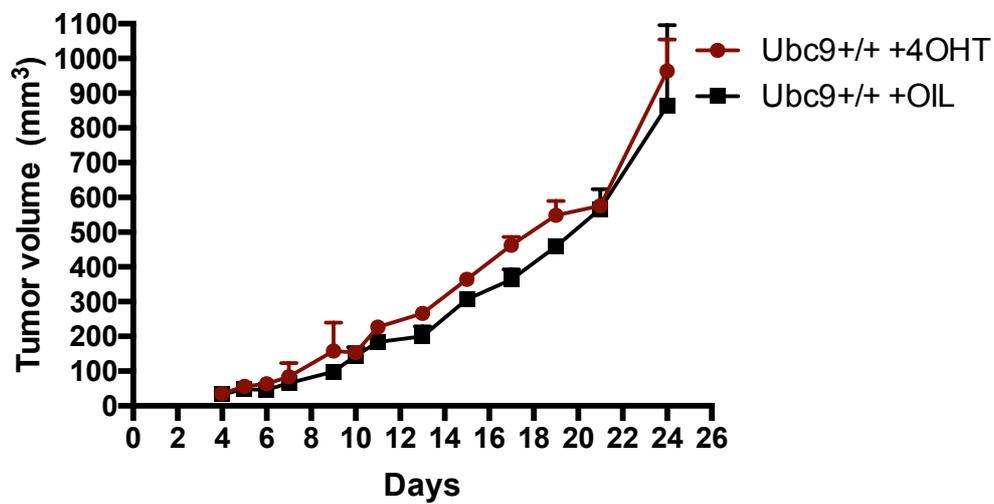
Soon after the subcutaneous injection with cells, we note the appearance of tumors which grew rapidly over the next days. Remarkably, tumors derived by  $Ubc9^{fl-}$  MEFs appeared faster than the wild-type ones, taking only 3 days after the injection. They presented also a faster growth, with the volume of the tumor exceeding  $500\text{mm}^3$  in only 8 days after the injections (Figure 24A). In contrast, the  $Ubc9^{+/+}$  MEFs derived tumors started appearing between 4-5 days after the injection and their volume didn't exceed  $110\text{mm}^3$  until the end of the experiment, 16 days after the injections (Figure 24B). We can clearly see the difference in the size of tumors between  $Ubc9^{fl-}$  and  $Ubc9^{+/+}$  MEFs during (Figure 24C) and at the end of the experiment (Figure 24D) with the heterozygous ones being constantly bigger. These results are concur with the *in vitro* ones discussed previously, and confirm that the heterozygous MEFs have a higher transformation capacity compared to the wild-type ones.

Once the first tumors appeared, after 3 days, we initiated the treatment with 4-OHT by intraperitoneal injections every 2 days. Mice that were used as control were injected with OIL every 2 days. No difference was observed in the tumor growth of *Ubc9*<sup>+/+</sup> MEFs with or without 4-OHT (Figure 24B). However, the growth of tumors derived from *Ubc9*<sup>f/-</sup> MEFs appears to be slightly slower when treated with 4-OHT (Figure 24A). Nevertheless, the knockout of *Ubc9* couldn't arrest the growth of the tumor until the end of experiment at 17 days when the mice reached the highest permitted tumor volume (Figure 24A). The highest permitted volume was set at 1000mm<sup>3</sup> after which the mice would be sacrificed. Surprisingly, the mice that had not received 4-OHT injection started dying before the tumor had reached the highest volume presenting signs of cachexia, with massive loss of total body mass, and all the mice had to be sacrificed between 12 and 14 days.



**Figure 24 Xenografts in nude mice.** **A:** Evolution of the volume of tumors derived by *Ubc9*<sup>f/-</sup> p53DN/HRasV12 transformed MEFs with 4-OHT treatment (red) or not (black). Mean  $\pm$  SD,  $n \geq 6$  per condition. **B:** Evolution of the volume of tumors derived by *Ubc9*<sup>+/+</sup> p53DN/HRasV12 transformed MEFs with 4-OHT treatment (red) or not (black). Mean  $\pm$  SD,  $n \geq 6$  per condition. **C:** Representative pictures of the evolution of the tumors 4 and 8 days after the injection in the same mouse. Left side: *Ubc9*<sup>+/+</sup> Right side: *Ubc9*<sup>f/-</sup> MEFs. **D:** Representative picture of the tumors at the end of the experiment. Left side: *Ubc9*<sup>+/+</sup> Right side: *Ubc9*<sup>f/-</sup> MEFs.

This would suggest that tumors heterozygous for *Ubc9* have a severe effect on the xenograft mice causing cachexia, which is not the case when we knockout *Ubc9* in the tumors, which seems to reduce the severity of the impact that the tumor growth has for the mice. To investigate whether it is genotype related and not a generic protective effect of the 4-OHT treatment and to evaluate the growth potential of the wild-type cells, we performed an experiment where we injected only wild-type MEFs in higher population, followed by 4-OHT treatment or not. In both conditions the mice reached the highest permitted tumor volume (1000mm<sup>3</sup>) at 24 days after injection with 2,8 million of *Ubc9*<sup>+/+</sup> p53DN/HRasV12 transformed MEFs and none of the mice presented signs of cachexia (Figure 25).



**Figure 25 Xenografts in nude mice.** Evolution of the volume of tumors derived by *Ubc9*<sup>+/+</sup> p53DN/HRasV12 transformed MEFs with 4-OHT treatment (red) or not (black). Mean  $\pm$  SD, n=2 per condition.

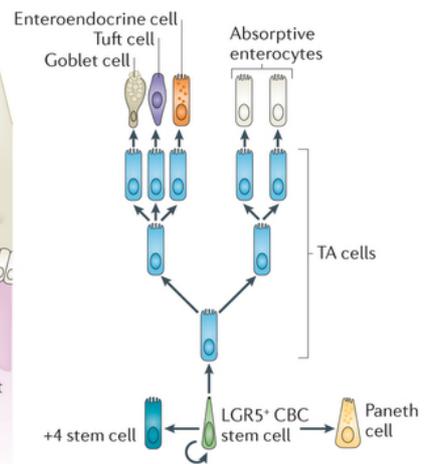
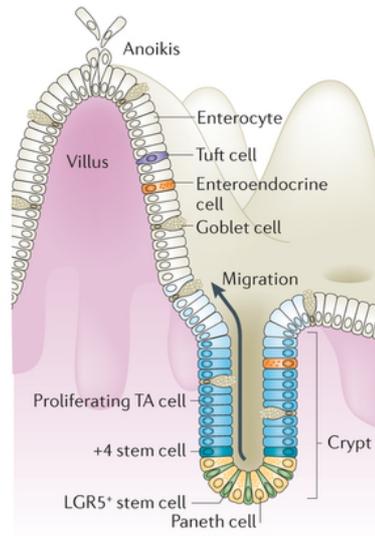
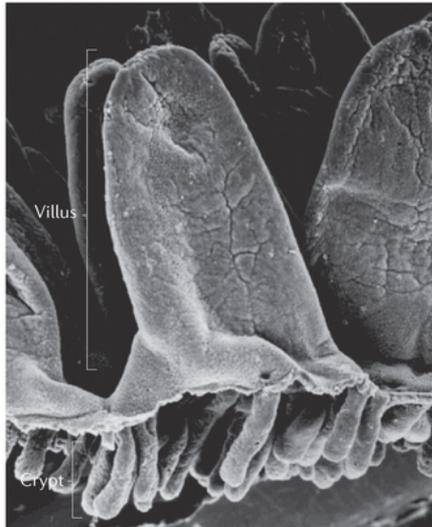
## ***In vivo* study of the implication of the SUMOylation in colorectal cancer**

Before we discuss the results of the different models used for the *in vivo* study of colorectal cancer it would be useful to briefly describe the structural organization of the intestine. The intestine is a complex tissue whose primary function is the digestion and absorption of nutrients and water. It consists of the small intestine and the colon. The small intestine consists of three regions, the duodenum, the jejunum and the ileum.

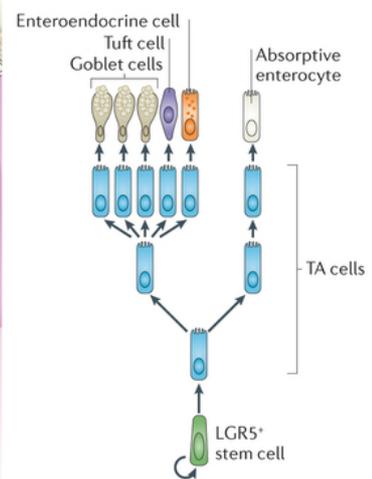
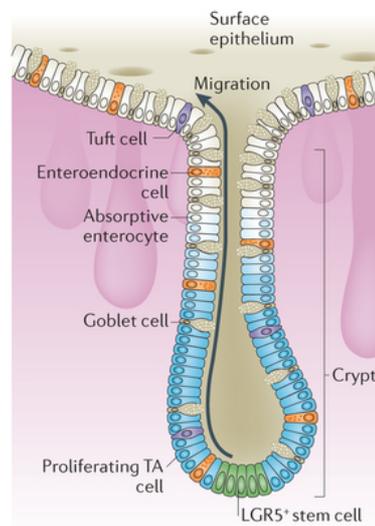
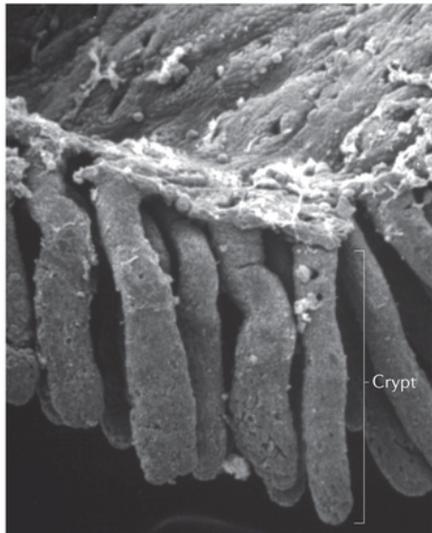
The small intestine is organized in villus and crypts. At the base of the crypts we find the LGR5+ (Leu-rich repeat-containing G protein-coupled receptor 5-expressing) stem cells which are intercalated with Paneth cells. These stem cells give rise to the rapidly proliferating transit-amplifying (TA) cells which in turn differentiate into the four different categories of functional cells of the villi, namely a) goblet cells, b) enterocytes, c) enteroendocrine cells and d) tuft cells that in turn replace the epithelial cells lost by anoikis. In the intestine there is a population of stem cells in the +4 position from the crypt base which are capable of restoring the LGR5+ cells in case of injury (Figure 26A). The intestinal epithelial turnover is very rapid, every 3-5 days.

The structural organization of the colon is similar to that of the intestine although it lacks villi and is only composed by crypts. At the base of the crypt we have the LGR5+ cells without Paneth cells this time. The LGR5+ cells generate the TA cells which in turn differentiate into the four cell types of the crypt (goblet, enterocytes, enteroendocrine and tuft cells) (Figure 26B). The epithelial turnover of the colon happens every 5-7 days.

**a Small intestine**



**b Colon**



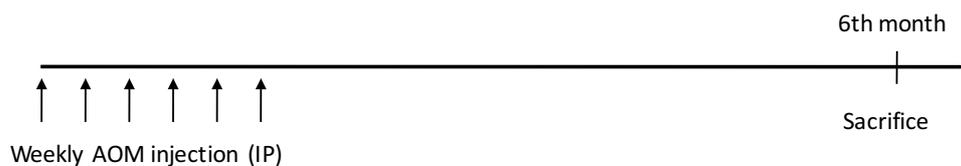
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**Figure 26 Structural organization of A: the small intestine and B: the colon (Barker 2014).**

## Role of *Ubc9* haploinsufficiency in the colorectal tumorigenesis.

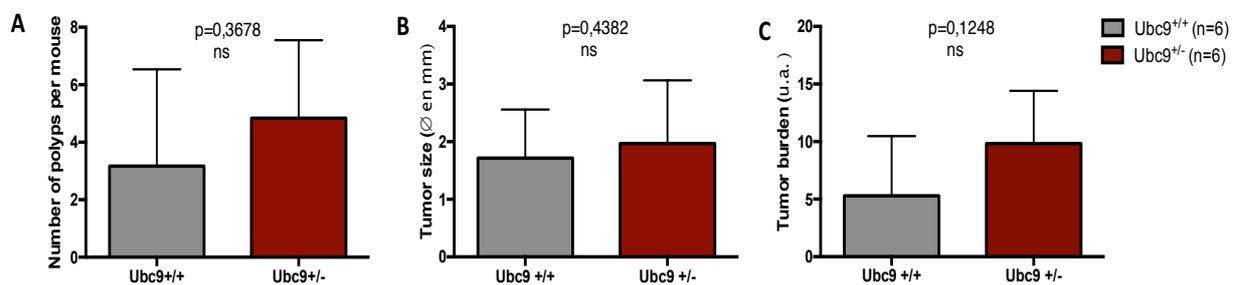
The oncogenesis can be induced by genetic or environmental causes. The study of the role of SUMOylation in a genetic model for colorectal cancer will be discussed later in this manuscript. To evaluate the role of SUMOylation in the colorectal carcinogenesis induced by a mutagenic agent, we injected azoxymethanin (AOM) in two groups of mice: the *Ubc9*<sup>+/-</sup> mice and the *Ubc9*<sup>+/+</sup> wild-type mice. The AOM is a precursor of MAM (MethylAzoxyMethanol), an alkylating agent of DNA, whose principal target is the methylation of the nucleotide guanine (Delker, McKnight and Rosenberg, 1998). The AOM has as principal target the colon making it a valuable model for the study of colorectal carcinogenesis in mice. A weekly injection of AOM during 6 weeks generates the formation of polyps principally in the distal part of colon 6 months after the beginning of the treatment (Figure 27).

### Carcinogenesis dependent on mutagenic agent



**Figure 27** Schematic representation of the AOM protocol for colorectal cancer.

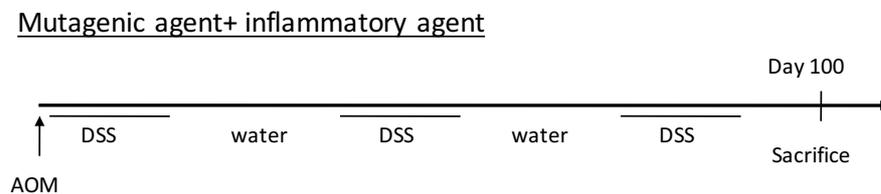
The low number of polyps induced doesn't reveal any statistical significant difference between the two genotypes in terms of number (Figure 28A), size (Figure 28B) or tumor burden (Figure 28C).



**Figure 28** The haploinsufficiency of *Ubc9* doesn't have a significant effect on the colorectal carcinogenesis induced by AOM. **A:** Number of polyps in the colon of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> mice after AOM treatment. Mean ± SD, n=6. **B:** Tumor size of the polyps in the colon of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> mice after AOM treatment. Mean ± SD, n=6. **C:** Tumor burden, as measured by the sum of the diameters of the total polyps per mouse in the colon of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> mice after AOM treatment. Mean ± SD, n=6 per genotype. p-value: Unpaired student's t-test.

## The haploinsufficiency of *Ubc9* reduces the colorectal carcinogenesis induced by the exposure to one alkylating and one inflammatory agent

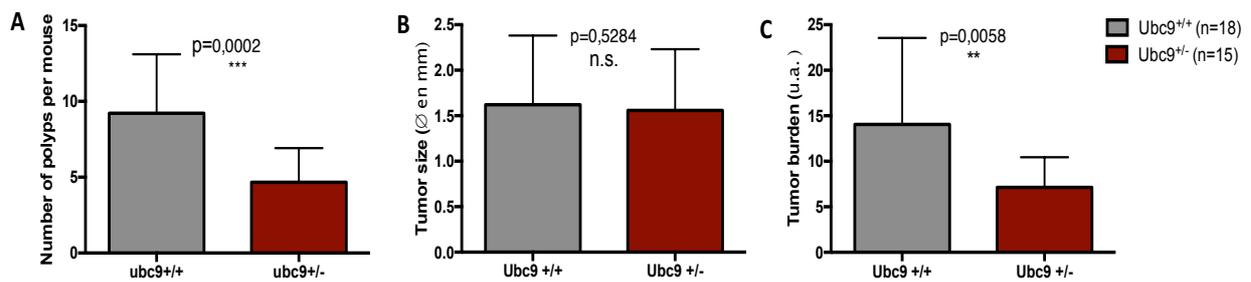
As the AOM alone is not enough to induce sufficient number of polyps and due to the long incubation time it requires, we used another model that combines a chemical and an inflammatory agent thereby inducing a stronger phenotype after three months of treatment. The protocol followed consists of a single injection of AOM in mice *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> followed by three rounds of one week of DSS (Dextran Sulfate) interrupted by two weeks of water between the rounds of DSS (Figure 29). This model has been frequently used in a lot of studies (Neufert, Becker and Neurath, 2007) and permits the development of higher number of polyps in the mice than AOM alone.



**Figure 29 Schematic representation of the AOM-DSS protocol for colorectal cancer.**

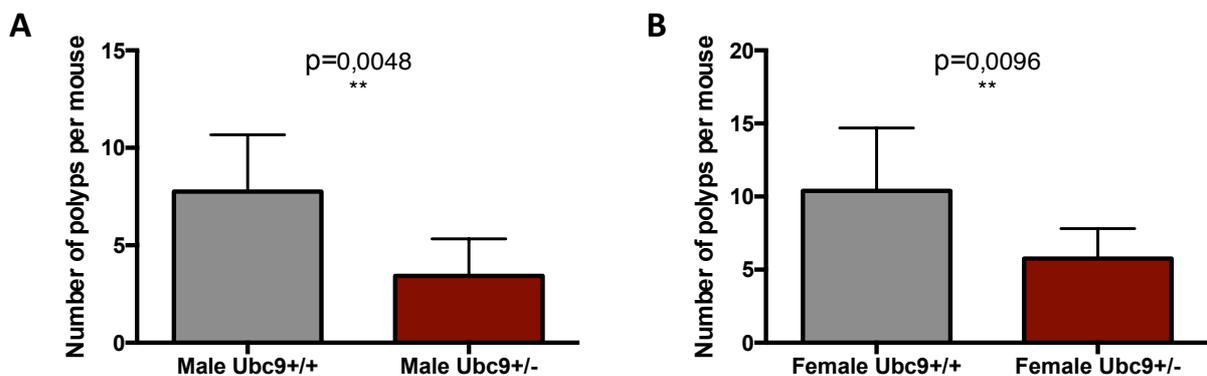
The intestine is a target of chronic inflammatory diseases such as Crohn disease or the ulcerative colitis. The inflammation linked to those diseases creates a favorable environment for the development of adenomas that progress to adenocarcinomas. The increased risk for the development of a colorectal carcinoma (CCR) in patients with chronic inflammatory diseases has been well known for almost a century (Andersen and Jess, 2013). As previous results in the lab have revealed an important role of SUMOylation in the control of the inflammatory transcriptional program, we decided to evaluate the role of SUMOylation in the oncogenesis promoted by inflammation.

Remarkably, the analysis of the colon tissue after AOM-DSS treatment, revealed a 2-fold decrease in the number of polyps developed in the *Ubc9*<sup>+/-</sup> mice when compared to the wild-type *Ubc9*<sup>+/+</sup> ones (Figure 30A). The tumor burden is also reduced in half in the *Ubc9* haploinsufficient mice (Figure 30C). However, no difference was observed regarding the size of polyps suggesting that it affects the initiation and not the progression of cancer (Figure 30B). Together these results demonstrate an important role of SUMOylation in the colorectal carcinogenesis induced by the exposure to one alkylating and one inflammatory agent.



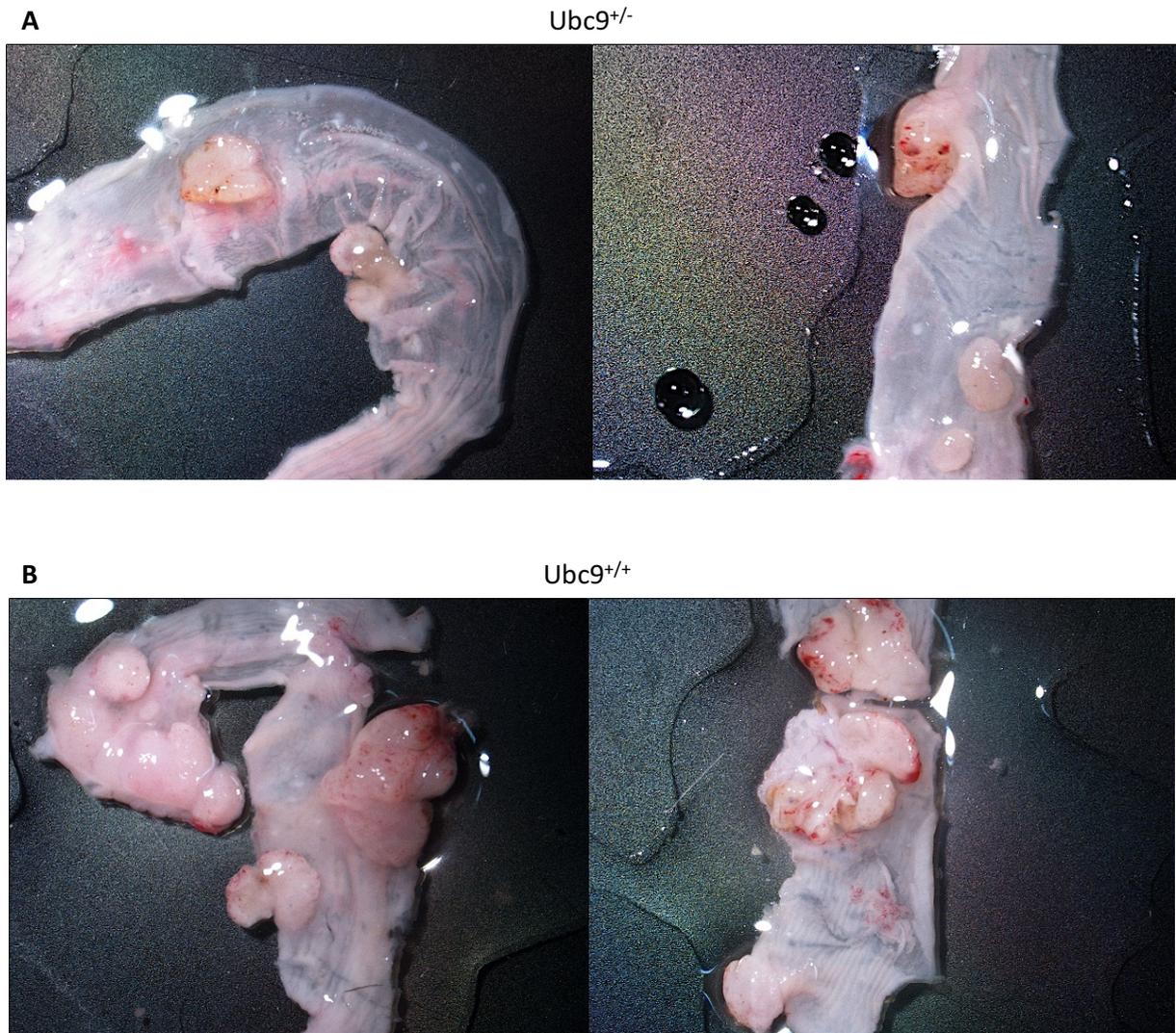
**Figure 30 The haploinsufficiency of *Ubc9* plays an important role in the colorectal carcinogenesis induced by AOM-DSS.** A: Number of polyps in the colon of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> mice after three months of AOM-DSS treatment. Mean ± SD, n≥15. B: Tumor size of the polyps in the colon of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> mice after three months of AOM-DSS treatment. Mean ± SD, n≥15. C: Tumor burden, as measured by the sum of the diameters of the total polyps per mouse in the colon of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> mice after three months of AOM-DSS treatment. Mean ± SD, n≥15 per genotype. p-value: Unpaired student's t-test.

To assess a possible effect of the gender in the prevalence of polyps, the number of polyps was evaluated based on the gender of mice between genotypes. We observe a highly significant difference of 2-fold change both in male and female between the different genotypes (Figure 31A, 31B).



**Figure 31 The gender plays no role in the colorectal carcinogenesis induced by AOM-DSS** A: Number of polyps in the colon of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> male mice respectively three months after AOM-DSS treatment. B: Number of polyps in the colon of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> female mice respectively three months after AOM-DSS treatment. Mean ± SD, n≥7 per sex and per genotype. p-value: Unpaired student's t-test.

The difference between the genotypes, with higher number of polyp prevalence in the *Ubc9<sup>+/+</sup>* mice is demonstrated in the representative images of the tumorigenesis in the colon of *Ubc9<sup>+/+</sup>* and *Ubc9<sup>+/-</sup>* mice after induction of tumorigenesis by AOM-DSS treatment (Figure 32).



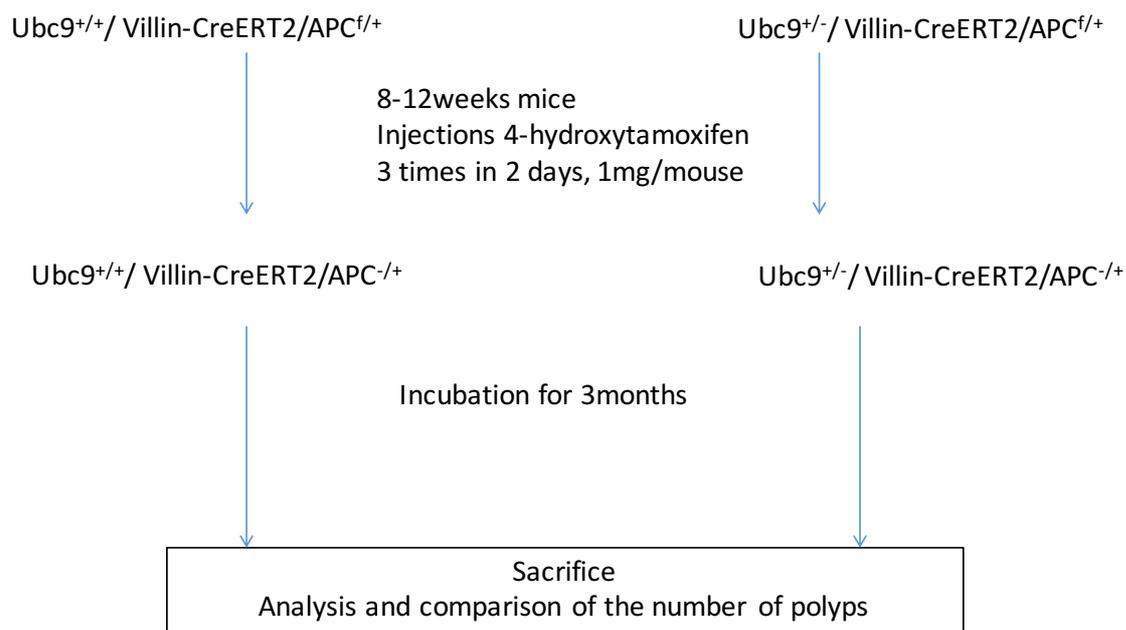
**Figure 32 Representative pictures of polyps in the colon of mice treated with AOM-DSS.** A Stereoscopic images of the colon of two male *Ubc9<sup>+/-</sup>* mice three months after the AOM-DSS treatment. B Stereoscopic images of the intestine of two male *Ubc9<sup>+/+</sup>* mice three months after AOM-DSS treatment.

## **The haploinsufficiency of Ubc9 promotes the colorectal carcinogenesis in the APC colorectal cancer model.**

In order to evaluate the oncogenic potential of the haploinsufficient cells for *Ubc9* *in vivo*, *Ubc9<sup>f/-</sup>* mice were crossed with an inducible model for intestinal cancer based on the deletion of an allele of the tumor suppressor APC (Adenomatous Polyposis Coli) leading to the loss of signaling of APC. This organ is of particular interest for us since SUMOylation was previously shown in the laboratory to play an important role in the intestinal homeostasis. The APC protein is involved in more than 80% of colorectal cancers in humans (Kinzler and Vogelstein, 1996; Cancer Genom Atlas, 2012). The individuals which are carriers of the mutation of the APC gene in the germinal line develop during the first decades of their life a Familial Adenomatous Polyposis (FAP) which is characterized by the appearance of numerous polyps (small abnormalities on the surface of the intestinal tract) that could evolve into colorectal cancer (Groden *et al.*, 1991; Joslyn *et al.*, 1991; Kinzler *et al.*, 1991; Nishisho *et al.*, 1991). The APC protein interacts with numerous partners and acts as a tumor suppressor through the regulation of different cellular processes including the proliferation, the apoptosis, the differentiation, the adhesion and the migration. APC is a key component of the canonical Wnt/b-catenin pathway and is crucial for the regulation of the Wnt pathway, notably by promoting the degradation of the  $\beta$ -catenin. The loss of function of APC leads to accumulation of  $\beta$ -catenin, modifying the transcriptional program of the cell, thus promoting its proliferation, survival and differentiation (McCartney and Näthke, 2008).

The crucial role of APC in the colorectal carcinogenesis has positioned it as a valuable tool for development of various genetic models to study colorectal cancer by inactivation of the APC protein (Zeineldin and Neufeld, 2013). We focused on the *Apc <sup>$\Delta$ 14/+</sup>* model in which mice develop a severe phenotype that mimics the human FAP disease (Colnot *et al.*, 2004). The genetic modification of these animals consists in the deletion of the exon 14 of the APC gene, which is crucial in its role as a tumor suppressor. However, the severe phenotype of this deletion makes the maintenance of the mice population difficult. In order to overcome this problem, we have crossed our *Ubc9<sup>+/-</sup>* mice with mice of which the exon 14 of the *Apc* gene is floxed with loxP sites (*Apc<sup>f</sup>*), expressing the *CreERT2* transgene under the *Villin* promoter gene, which is specific for the intestine. This gave rise to the generation of mice *Ubc9<sup>+/-</sup>*

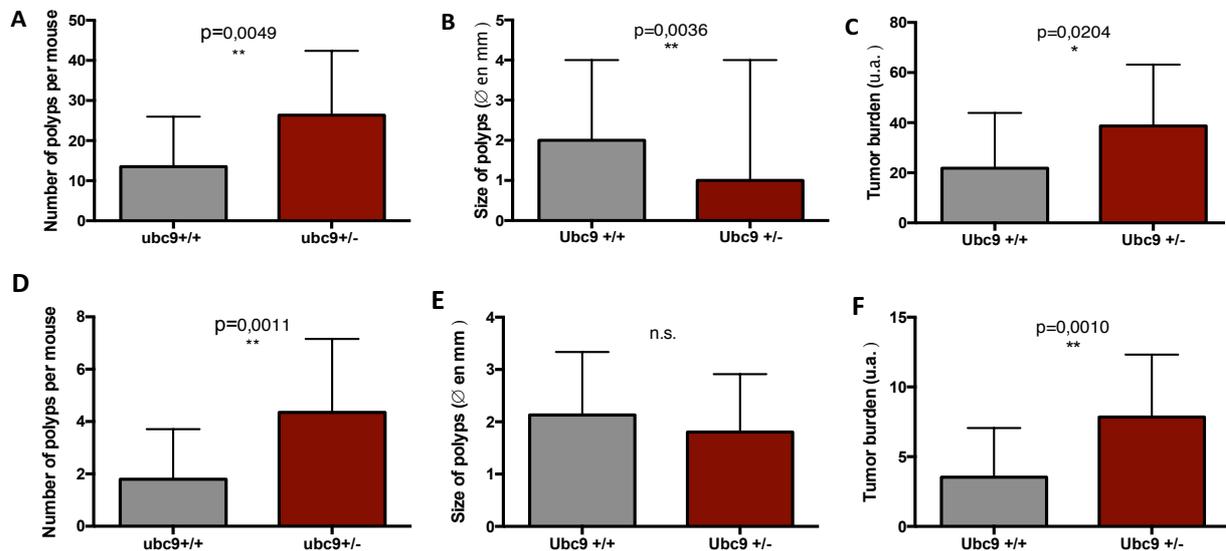
*/Villin-CreERT2/Apc<sup>f/+</sup>* mice. The APC<sup>f</sup> allele was previously described by Andreu, *et al.* (Andreu *et al.*, 2005). The intraperitoneal injection of mice with 4-hydroxytamoxifen permits the activation of the Cre-recombinase, expressed only in the intestine, and consequently the tissue-specific recombination of the *Apc* gene, recreating as a result the *Apc<sup>Δ14</sup>* allele. Based on those data, our lab established an experimental protocol permitting the generation of intestinal polyps in *Apc<sup>f/+</sup>* mice after the injection of 4-OHT at the age of 8-12 weeks and the analysis of the intestine three months after the injections (Figure 33).



**Figure 33 Experimental design used for the study of the role of SUMOylation in the colorectal carcinogenesis induced by loss of APC.**

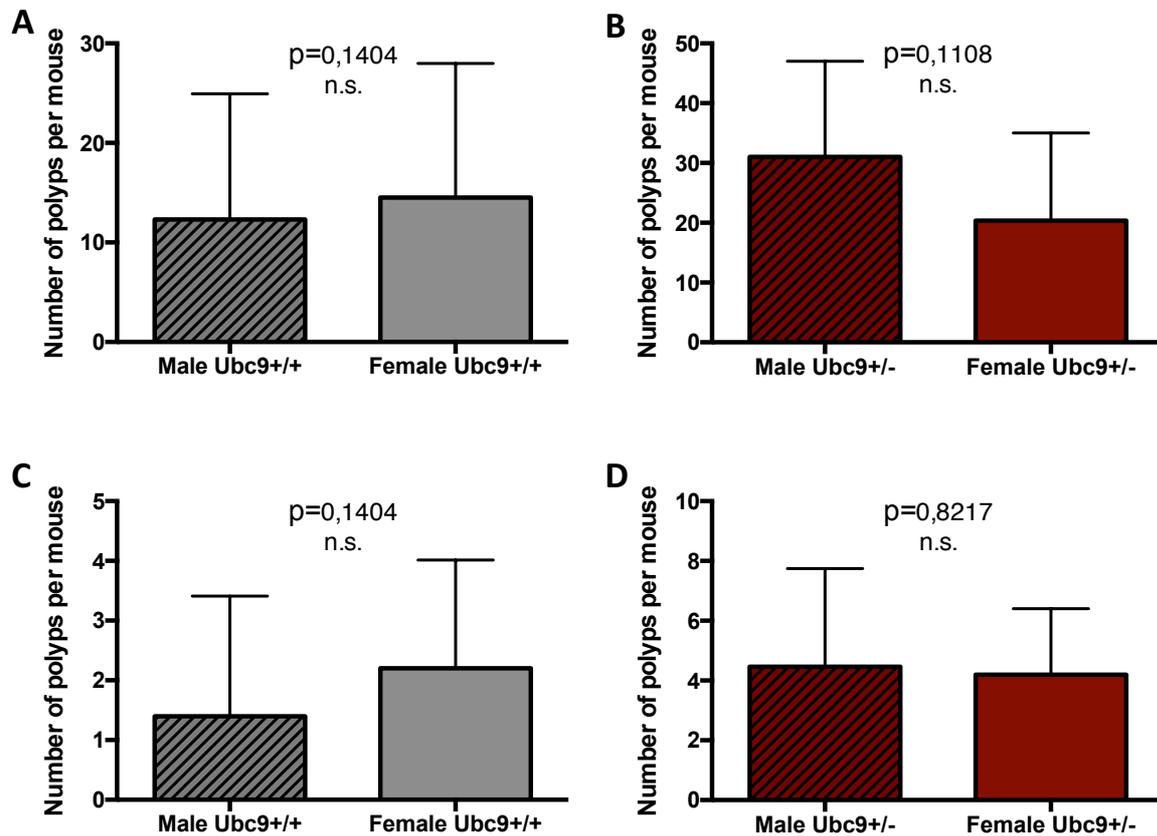
More than 20 mice per genotype (*Ubc9<sup>+/+</sup>/Villin-CreERT2/Apc<sup>f/+</sup>* and *Ubc9<sup>+/-</sup>/Villin-CreERT2/Apc<sup>f/+</sup>*) were used to analyze the role of SUMOylation in the intestinal tumorigenesis induced by the loss of *Apc*. Remarkably, the number of intestinal polyps was significantly (2-fold) higher in *Ubc9<sup>+/-</sup>* mice when compared to the wild-type ones (Figure 34A). Likewise, the tumor burden is two times higher in *Ubc9<sup>+/-</sup>* mice when compared to the wild-type ones (Figure 34C). Tumor size, by contrast, was higher in *Ubc9<sup>+/+</sup>* mice (Figure 34B) suggesting that tumor initiation, rather than tumor growth, is affected by *Ubc9* haploinsufficiency. Therefore, reducing the UBC9 level to a half-dose significantly enhances the incidence of intestinal tumors in this setting.

Similar findings were obtained in the colon with no difference in the size of polyps (Figure 34E) despite the higher number of polyps (Figure 34D) and tumor burden (Figure 33F) in *Ubc9*<sup>+/-</sup> mice. Together all these data demonstrate that SUMOylation plays an important role in the suppressive process of the genetically induced colorectal carcinogenesis.



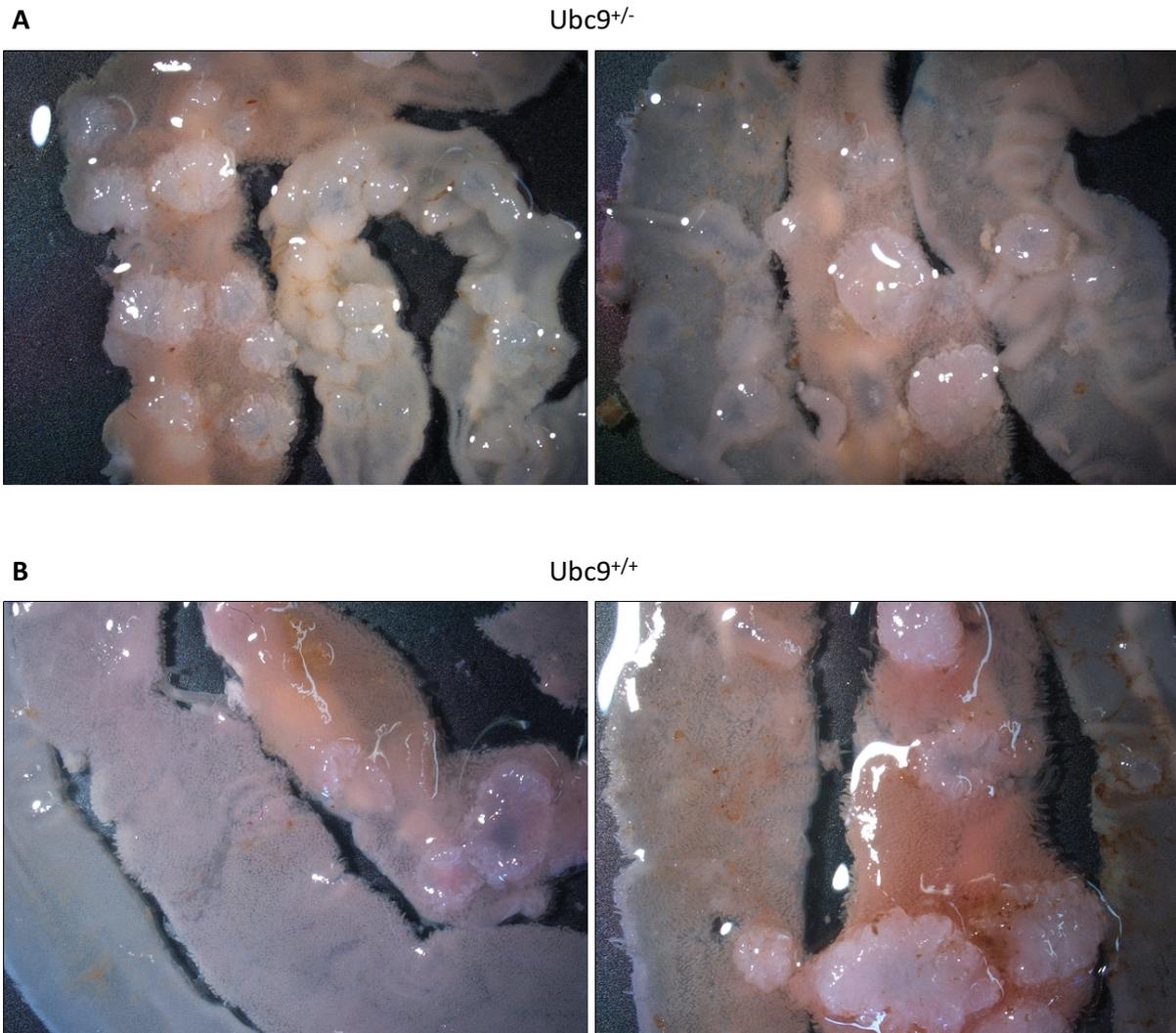
**Figure 34 The haploinsufficiency of *Ubc9* promotes colorectal tumorigenesis induced by the loss of APC.** **A:** Number of polyps in the intestine of *Ubc9*<sup>+/+</sup>/*Villin-CreERT2/Apc*<sup>f/+</sup> and *Ubc9*<sup>+/-</sup>/*Villin-CreERT2/Apc*<sup>f/+</sup> mice three months after 4-OHT injections. Mean ± SD, n≥21. **B:** Tumor size of the polyps in the intestine of *Ubc9*<sup>+/+</sup>/*Villin-CreERT2/Apc*<sup>f/+</sup> and *Ubc9*<sup>+/-</sup>/*Villin-CreERT2/Apc*<sup>f/+</sup> mice after 4-OHT injections. Mean ± SD, n≥21. **C:** Tumor burden, as measured by the sum of the diameters of the total polyps per mouse in the intestine of *Ubc9*<sup>+/+</sup>/*Villin-CreERT2/Apc*<sup>f/+</sup> and *Ubc9*<sup>+/-</sup>/*Villin-CreERT2/Apc*<sup>f/+</sup> mice three months after 4-OHT injections. Mean ± SD, n≥21 per genotype. p-value: Unpaired student's t-test. **D:** Number of polyps in the colon of *Ubc9*<sup>+/+</sup>/*Villin-CreERT2/Apc*<sup>f/+</sup> and *Ubc9*<sup>+/-</sup>/*Villin-CreERT2/Apc*<sup>f/+</sup> mice three months after 4-OHT injections. Mean ± SD, n≥21. **E:** Tumor size of the polyps in the colon of *Ubc9*<sup>+/+</sup>/*Villin-CreERT2/Apc*<sup>f/+</sup> and *Ubc9*<sup>+/-</sup>/*Villin-CreERT2/Apc*<sup>f/+</sup> mice three months after 4-OHT injections. Mean ± SD, n≥21. **F:** Tumor burden, as measured by the sum of the diameters of the total polyps per mouse in the colon of *Ubc9*<sup>+/+</sup>/*Villin-CreERT2/Apc*<sup>f/+</sup> and *Ubc9*<sup>+/-</sup>/*Villin-CreERT2/Apc*<sup>f/+</sup> mice three months after 4-OHT injections. Mean ± SD, n≥21 per genotype. p-value: Unpaired student's t-test.

To assess the possible effect of gender in the prevalence of polyps, the number of polyps was evaluated based on the gender of mice of the same genotype. The gender of the mice doesn't make any difference in the prevalence of polyps between mice of the same genotype in the intestine (Figure 35A, 35B) nor in colon (Figure 35C, 35D).



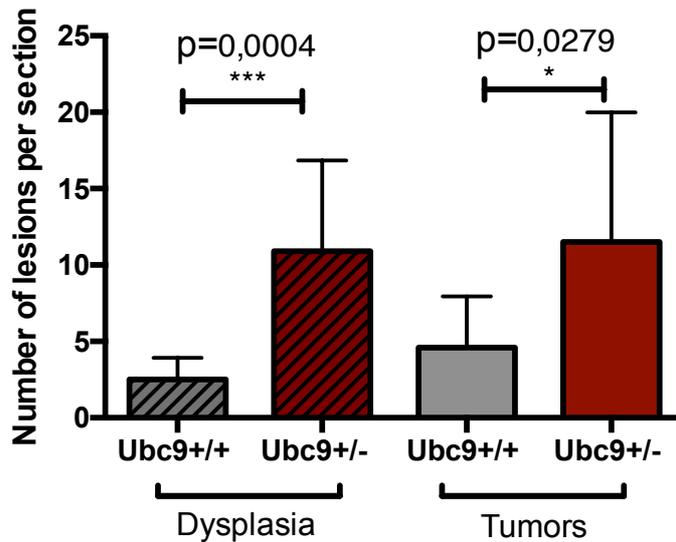
**Figure 35 The gender plays no role in the colorectal carcinogenesis induced by the loss of APC.** **A:** Number of polyps in the intestine of *Ubc9*<sup>+/+</sup>/*Villin-CreERT2*/*Apc*<sup>f/+</sup> male and female mice respectively. **B:** Number of polyps in the intestine of *Ubc9*<sup>+/-</sup>/*Villin-CreERT2*/*Apc*<sup>f/+</sup> male and female mice respectively. **C:** Number of polyps in the colon of *Ubc9*<sup>+/+</sup>/*Villin-CreERT2*/*Apc*<sup>f/+</sup> male and female mice respectively. **D:** Number of polyps in the colon of *Ubc9*<sup>+/-</sup>/*Villin-CreERT2*/*Apc*<sup>f/+</sup> male and female mice respectively. Mice were sacrificed three months after 4-OHT injections. Mean  $\pm$  SD,  $n \geq 10$  per sex and per genotype. p-value: Unpaired student's t-test.

Representative images of the tumorigenesis in the intestines of *Ubc9<sup>+/-</sup>/Villin-CreERT2/Apc<sup>fl/+</sup>* and *Ubc9<sup>+/-</sup>/Villin-CreERT2/Apc<sup>fl/+</sup>* mice after the loss of APC are presented in the Figure 36. We can clearly see the difference of higher number of polyp prevalence in the *Ubc9<sup>+/-</sup>* mice.



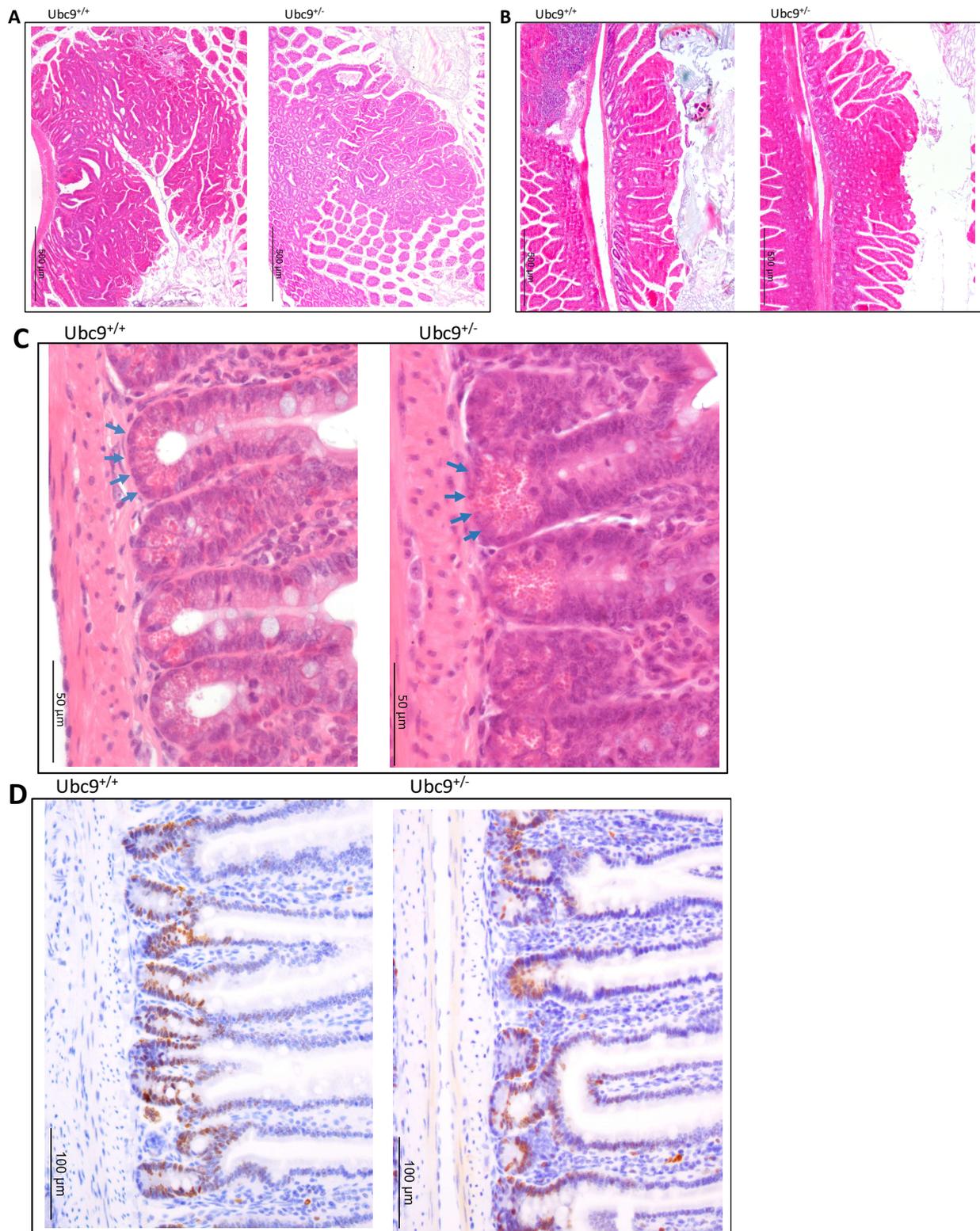
**Figure 36 Representative pictures of intestinal polyps induced by the loss of APC.**  
**A:** Stereoscopic images of the intestine of two female *Ubc9<sup>+/-</sup>/Villin-CreERT2/Apc<sup>fl/+</sup>* mice three months after the 4-OHT injections. **B:** Stereoscopic images of the intestine of two female *Ubc9<sup>+/+</sup>/Villin-CreERT2/Apc<sup>fl/+</sup>* mice three months after the 4-OHT injections.

Histological analysis using the intestine of *Ubc9<sup>+/+</sup>/Villin-CreERT2/Apc<sup>fl/+</sup>* and *Ubc9<sup>+/-</sup>/Villin-CreERT2/Apc<sup>fl/+</sup>* mice was performed to confirm those results and analyze the severity of the lesions. Remarkably, and in line with the previous results, the number of intestinal dysplasia and tumors was significantly (4,3- and 2,5-fold respectively) higher in *Ubc9<sup>+/-</sup>* mice when compared to their wild-type ones (Figure 37).

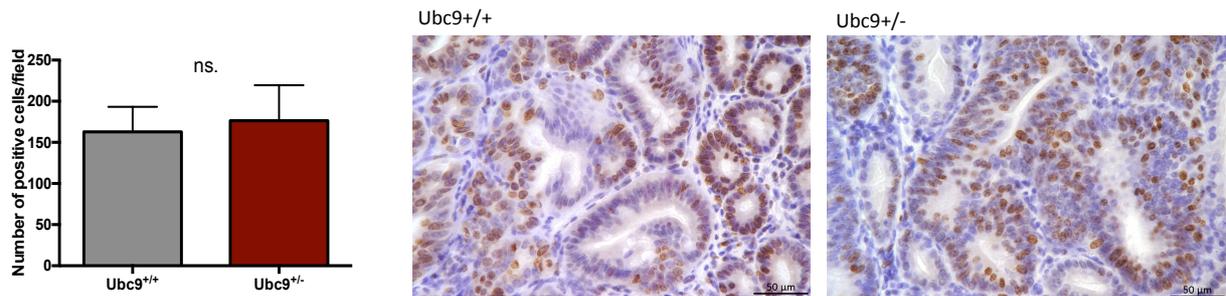


**Figure 37 Histological analysis of H&E stained intestines reveals increased tumorigenesis in *Ubc9<sup>+/-</sup>* mice.** Incidence of intestinal dysplasia and tumors in the intestine of *Ubc9<sup>+/+</sup>/Villin-CreERT2/Apc<sup>fl/+</sup>* and *Ubc9<sup>+/-</sup>/Villin-CreERT2/Apc<sup>fl/+</sup>* mice 3 months after 4-OHT injections. Mean ± SD, n=10 per genotype. p-value: Unpaired student's t-test.

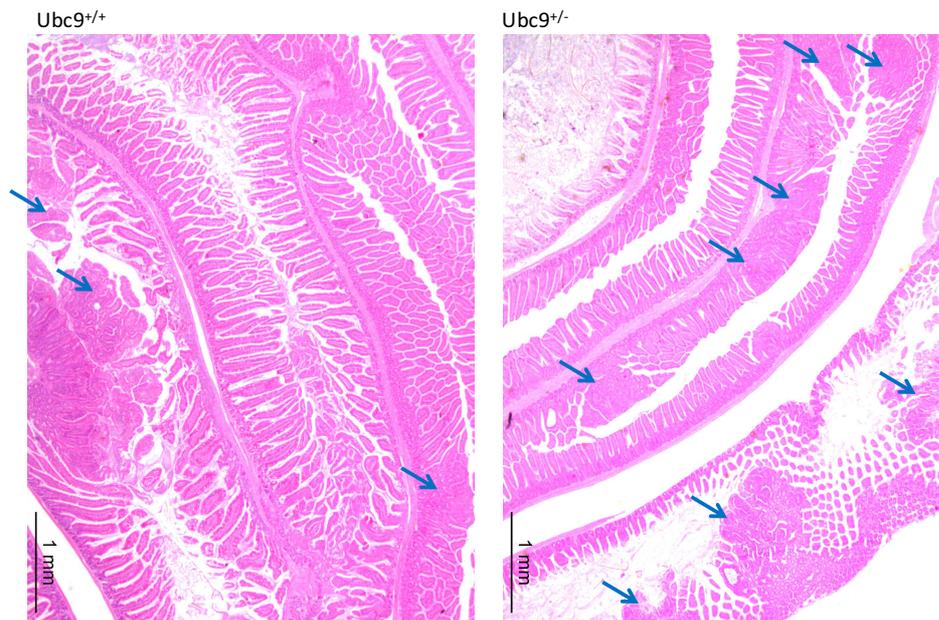
In addition, the histological analysis of the tumors revealed no difference in the nature and morphology/organization between the different genotypes (Figure 38A, 38B). Moreover, the number and morphology of paneth cells is the same in *Ubc9<sup>+/+</sup>* and *Ubc9<sup>+/-</sup>* mice (Figure 38C). Staining with Ki67, a marker of cell proliferation, revealed no significant difference in normal intestinal tissue (Figure 38D) and tumors (Figure 39) between the different genotypes. Finally, in concurrence with our data, the histological analysis revealed higher density of tumors in the *Ubc9<sup>+/-</sup>* mice comparing to the wild-type ones (Figure 40).



**Figure 38 Same nature and morphology/organisation for all mice.** Histological analysis of H&E stained intestines of *Ubc9*<sup>+/+</sup>/*Villin-CreERT2/Apc*<sup>f/+</sup> and *Ubc9*<sup>+/-</sup>/*Villin-CreERT2/Apc*<sup>f/+</sup> mice 3 months after 4-OHT injections. **A:** Same nature of tumors: epithelial **B:** Same morphology/organization: early stages, dysplasia and small adenoma **C:** Visualization and counting of Paneth cells revealed no difference between *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> mice **D:** Ki67 staining revealed no difference in the cell proliferation between normal crypts of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> mice.

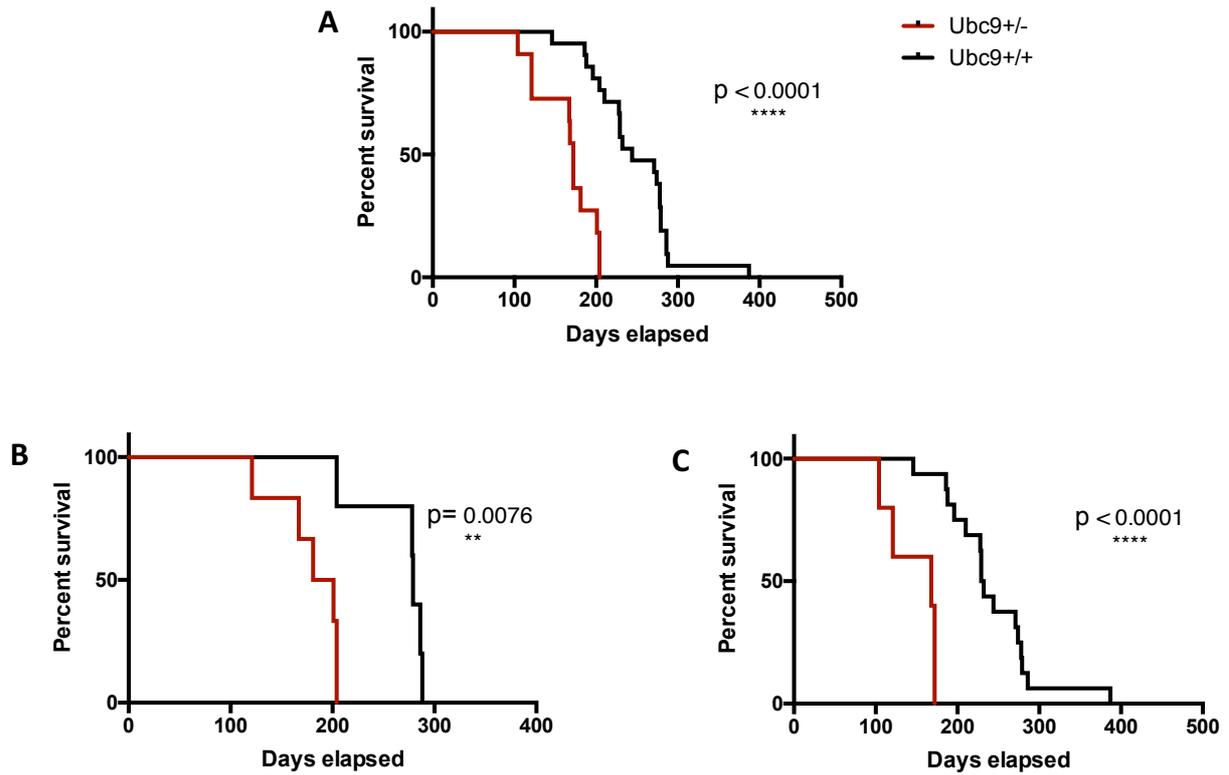


**Figure 39** No difference in cell proliferation between the tumors of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> mice. Number of positive cells per field after Ki67 staining in 40X magnification. Mean  $\pm$  SD, *Ubc9*<sup>+/+</sup>: n=16 tumors, *Ubc9*<sup>+/-</sup>: n=28 tumors. p-value: Unpaired student's t-test.



**Figure 40** Higher density of tumors in *Ubc9*<sup>+/-</sup> mice. Histological analysis of H&E stained intestines of *Ubc9*<sup>+/+</sup>/Villin-CreERT2/*Apcf*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup>/Villin-CreERT2/*Apcf*<sup>+/+</sup> mice 3 months after 4-OHT injections.

Finally, the survival of the mice was analyzed over the period of 13 months. The *Ubc9*<sup>+/-</sup> mice began to die at 3,5 months and all of the mice were dead at 6,5 months, with evident signs of rectal bleeding and anemia. On the other hand, the *Ubc9*<sup>+/+</sup> mice started dying at 6 months with the rest of them (except of one) having deceased at 10 months (Figure 41A). No difference was observed based on gender between the different genotypes (Figure 41B, 41C). This demonstrates that the *Ubc9* haploinsufficient mice have significantly reduced survival expectancy when compared to the wild-type ones.



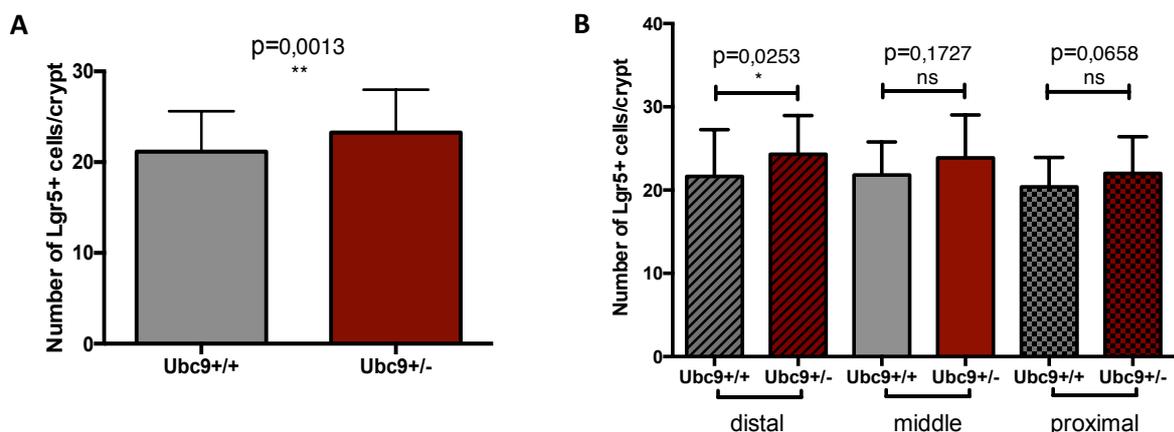
**Figure 41** The haploinsufficiency of *Ubc9* reduces the survival expectancy of mice with colorectal cancer induced by the loss of APC. **A:** Survival of *Ubc9*<sup>+/+</sup>/*Villin-CreERT2/Apc*<sup>f/f</sup> and *Ubc9*<sup>+/-</sup>/*Villin-CreERT2/Apc*<sup>f/f</sup> mice after 4-OHT injections. n=11. **B:** Survival of male *Ubc9*<sup>+/+</sup>/*Villin-CreERT2/Apc*<sup>f/f</sup> and *Ubc9*<sup>+/-</sup>/*Villin-CreERT2/Apc*<sup>f/f</sup> mice after 4-OHT injections. *Ubc9*<sup>+/-</sup>: n=5 *Ubc9*<sup>+/+</sup>: n=6. **C:** Survival of female APC<sup>+/+</sup> mice after 4-OHT injections. *Ubc9*<sup>+/-</sup>: n=6 *Ubc9*<sup>+/+</sup>: n=5. p-value: Unpaired student's t-test.

## Higher number of Lgr5+ stem cells in the *Ubc9*<sup>+/-</sup> mice

The impact of loss of one *Ubc9* allele on normal Lgr5<sup>+</sup> stem cells was assessed in the healthy intestinal epithelium from *Ubc9*<sup>+/+</sup>/Lgr5-EGFP-IRES-CreERT2 and *Ubc9*<sup>+/-</sup>/Lgr5-EGFP-IRES-CreERT2 mice. The number of Lgr5<sup>+</sup> stem cells per crypt, as assessed by GFP staining, was evaluated in *Ubc9*<sup>+/-</sup> and *Ubc9*<sup>+/+</sup> mice. A striking observation occurred from the evaluation of the number of Lgr5<sup>+</sup> cells with the *Ubc9*<sup>+/-</sup> mice presenting consistently two Lgr5<sup>+</sup> cells more than the wild-type ones. More specifically, we observe a mean of 21 Lgr5<sup>+</sup> cells in the *Ubc9*<sup>+/+</sup> mice against 23 in the *Ubc9*<sup>+/-</sup> mice (Figure 42A).

When focusing on the different parts of the intestine we notice a statistically significant difference in the distal part and a clear tendency in the middle and distal parts of the intestine (Figure 42B). Since the number of stem cells is low, increasing the number of mice studied will most likely increase the statistical significance in every part of the intestine. Analysis of more mice are currently being undertaken to increase the number of crypts analyzed.

Therefore, as it has been shown that the crypt stem cells are the cells of origin of colorectal cancer (Barker *et al.*, 2009), the increased number of stem cells that we observe in the entire intestine means an increased number of potential tumor-initiating cells, which could explain the increased tumorigenesis in the *Ubc9*<sup>+/-</sup> mice induced by APC.



**Figure 42 Higher number of Lgr5+ cells in the intestine of *Ubc9* haploinsufficient mice.**  
**A:** Number of Lgr5<sup>+</sup> cells per crypt in the entire small intestine of *Ubc9*<sup>+/+</sup>/Lgr5-EGFP-IRES-CreERT2 and *Ubc9*<sup>+/-</sup>/Lgr5-EGFP-IRES-CreERT2 mice. The intestines were fixed following the PLP-OCT protocol for maintenance of the fluorescence and GFP positive cells were counted by electronic microscopy. *Ubc9*<sup>+/+</sup>: n=99 crypts, 3mice, *Ubc9*<sup>+/-</sup>: n=102 crypts,

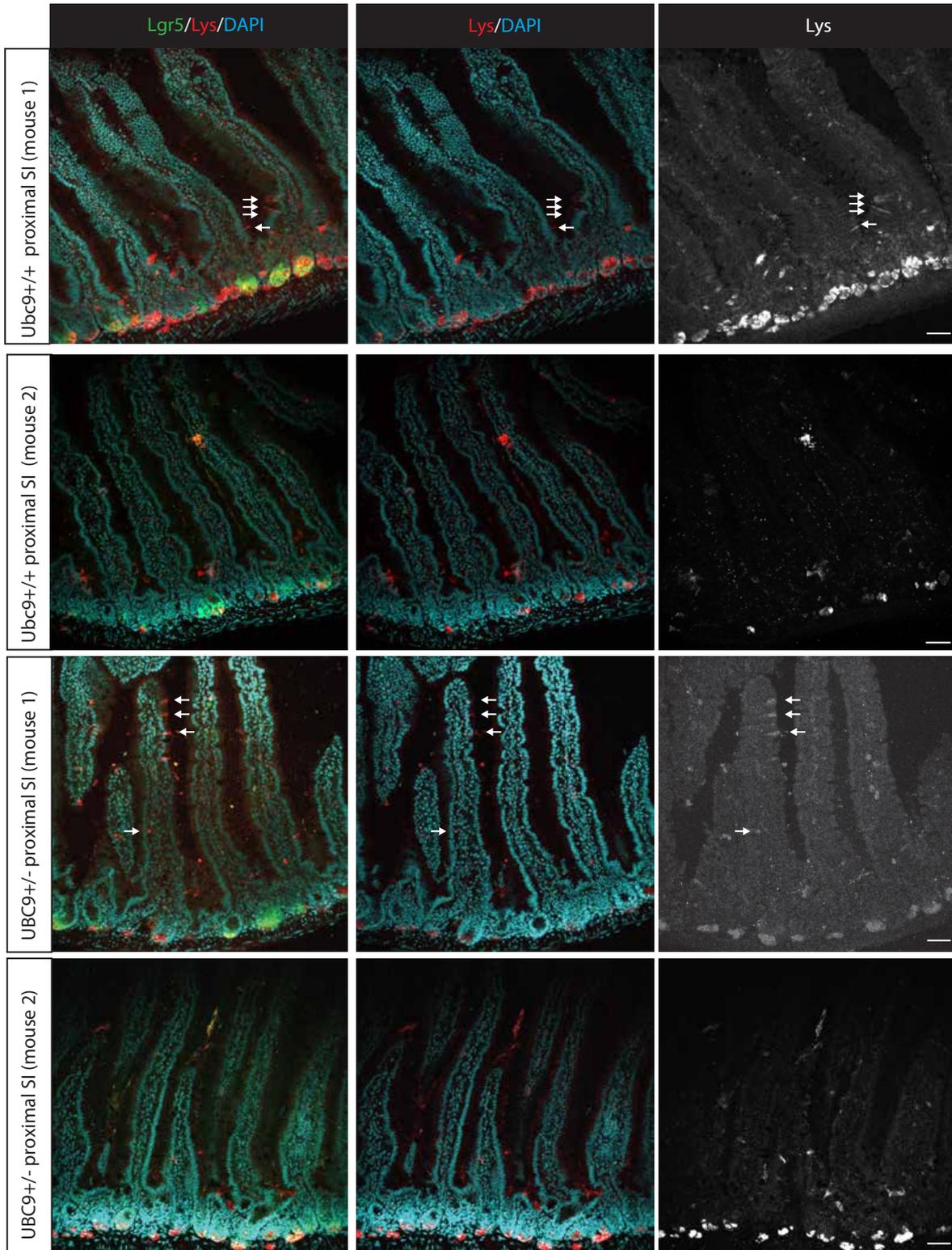
4mice. p-value: Unpaired student's t-test. **B:** Number of Lgr5+ cells per crypt in the distal middle and proximal parts of the intestine of *Ubc9<sup>+/+</sup>/Lgr5-EGFP-IRES-CreERT2* and *Ubc9<sup>+/-</sup>/Lgr5-EGFP-IRES-CreERT2* mice. The intestines were fixed following the PLP-OCT protocol for maintenance of the fluorescence and GFP positive cells were counted by electronic microscopy. Distal part: *Ubc9<sup>+/+</sup>*: n=35 crypts, 3mice, *Ubc9<sup>+/-</sup>*: n=43 crypts, 4mice. Middle part: *Ubc9<sup>+/+</sup>*: n=22 crypts, 3mice, *Ubc9<sup>+/-</sup>*: n=16 crypts, 4mice. Proximal part: *Ubc9<sup>+/+</sup>*: n=42 crypts, 3mice, *Ubc9<sup>+/-</sup>*: n=43 crypts, 4mice. p-value: Unpaired student's t-test.

Previous studies in the lab have shown that total loss of UBC9 results in mislocalized paneth cells, with random distribution through crypts and villi (Demarque *et al.*, 2011). In order to assess the effect of half dose of UBC9 in the niche, the intestine of 2 mice of each genotype was stained with an anti-lysozyme antibody to label the paneth cells. Furthermore, since proximodistal gradient of effects had been observed in total loss of UBC9, both proximal and distal small intestine was analyzed in each mouse.

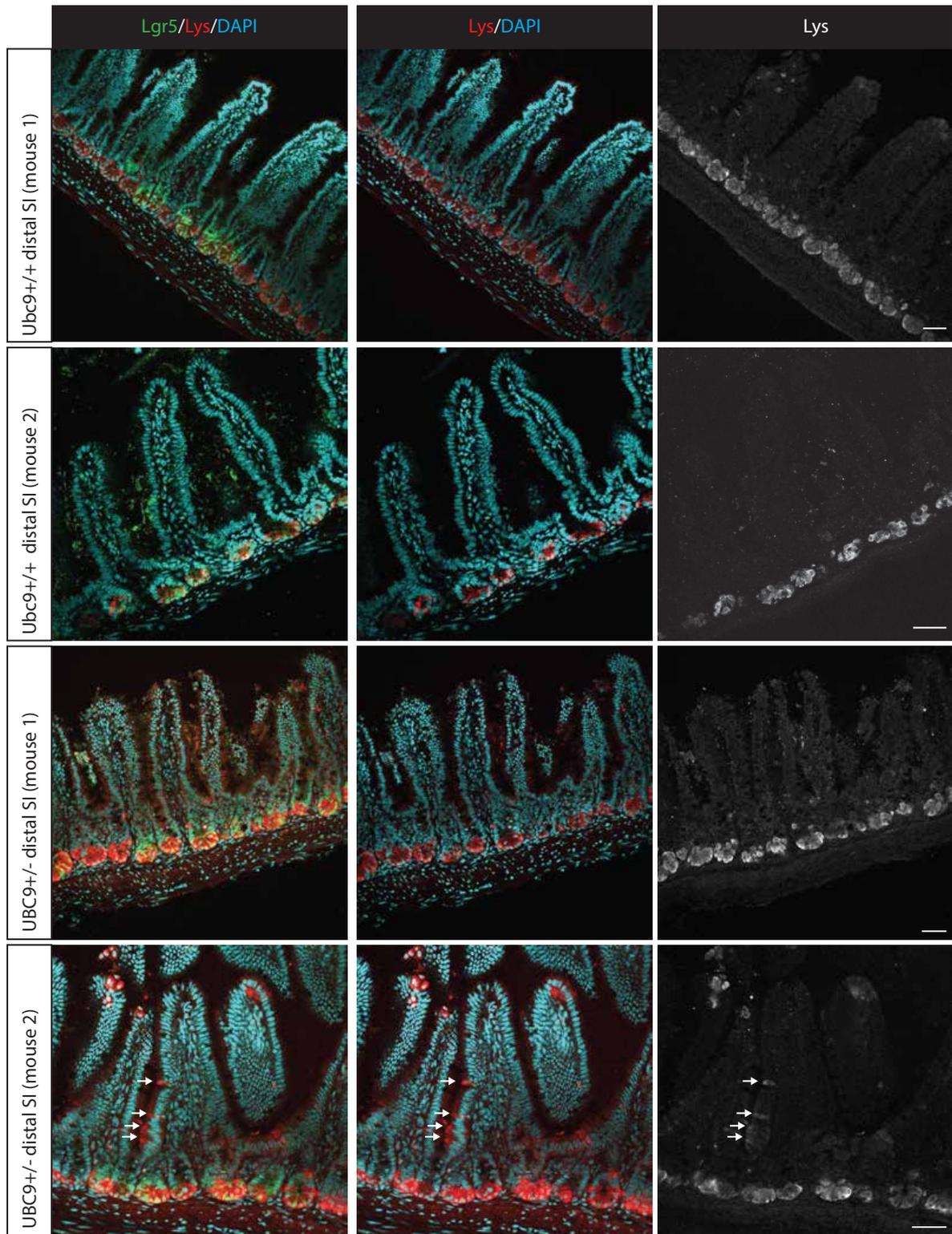
In proximal SI: paneth cells of both UBC9<sup>+/+</sup> and UBC9<sup>+/-</sup> are nicely located at the expected place at the base of the crypt and for both genotypes some lysozyme-positive cells are found higher up the villus (Figure 43)

In distal SI: for both UBC9<sup>+/+</sup> and UBC9<sup>+/-</sup> paneth cells are nicely located at the expected place at the base of the crypt and some lysozyme-positive cells are found higher up the villi of some crypts in UBC9<sup>+/-</sup> tissue (Figure 44)

These results suggest that there is no obvious change in the organisation of paneth cells in the heterozygous mice compared to the wild-type ones.



**Figure 43** Images of crypts in the proximal part of the small intestine of *Ubc9<sup>+/+</sup>/Lgr5-EGFP-IRES-CreERT2* and *Ubc9<sup>+/-</sup>/Lgr5-EGFP-IRES-CreERT2* mice. The intestines were assessed by electronic microscopy after staining with DAPI and lysozyme. Arrows point at lysozyme positive cells found higher up the villus.



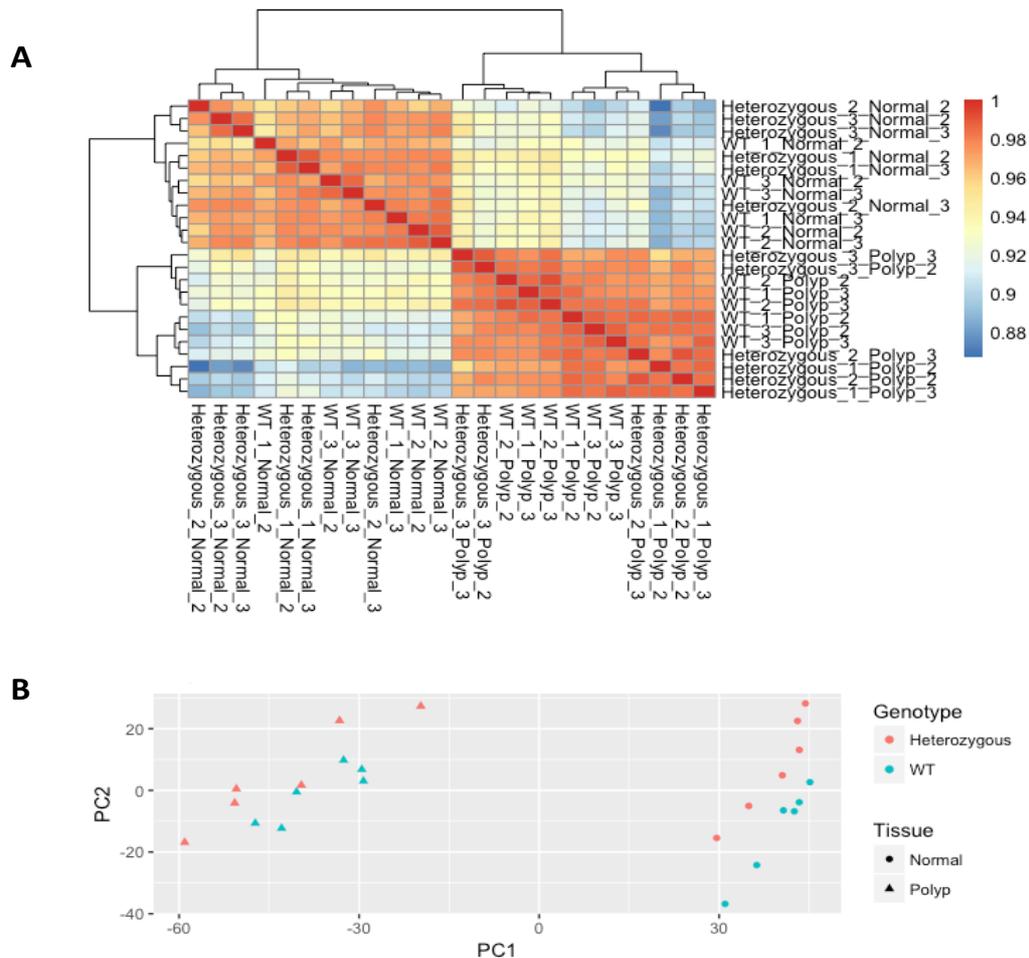
**Figure 44** Images of crypts in the distal part of the small intestine of *Ubc9<sup>+/+</sup>/Lgr5-EGFP-IRES-CreERT2* and *Ubc9<sup>+/-</sup>/Lgr5-EGFP-IRES-CreERT2* mice. The intestines were assessed by electronic microscopy after staining with DAPI and lysozyme. Arrows point at lysozyme positive cells found higher up the villus.

## **Transcriptomic analysis**

To assess the transcriptomic profile of the tumors and normal tissue between the different genotypes, *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> mice, affymetrix analysis was performed from samples collected three months after the treatment with AOM-DSS and loss of APC.

### **Transcriptomic analysis of samples derived from APC depletion**

Two different samples of polyps and surrounding normal tissue per mouse were analyzed from three different mice. As expected the transcriptional profile is different between polyps and normal tissue, with the normal tissue of heterozygous and wild-type mice clustering together and, subsequently, the polyps of heterozygous and wild-type mice clustering together as well (Figure 45). However, when looking into the genotype, the clustering is not as clear, as heterozygous and wild-type samples seem to be very similar both in normal tissue and in polyps (Figure 45).

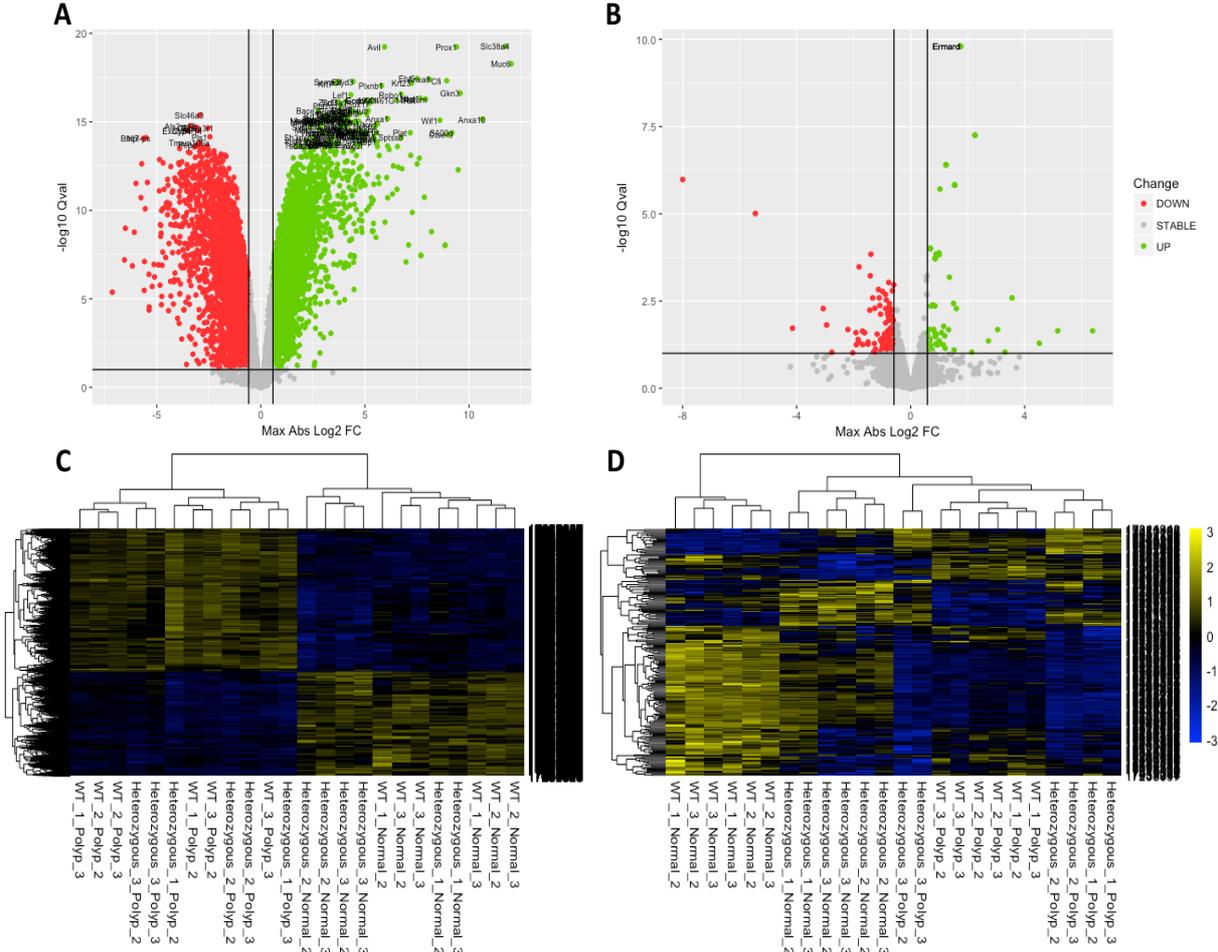


**Figure 45 Clustering after normalization of data for samples collected after APC loss.**

**A:** Heatmap after RMA using Ward's distance. In the Figure we have first the genotype heterozygous or wild-type (WT) then the number of the mouse, 1,2 or 3, then the tissue normal or polyp, and finally the sample numbered 2 or 3 for the two different samples analyzed per mouse **B:** PCA analysis after RMA. WT: *Ucc9<sup>+/+</sup>*, Heterozygous: *Ubc9<sup>+/-</sup>*

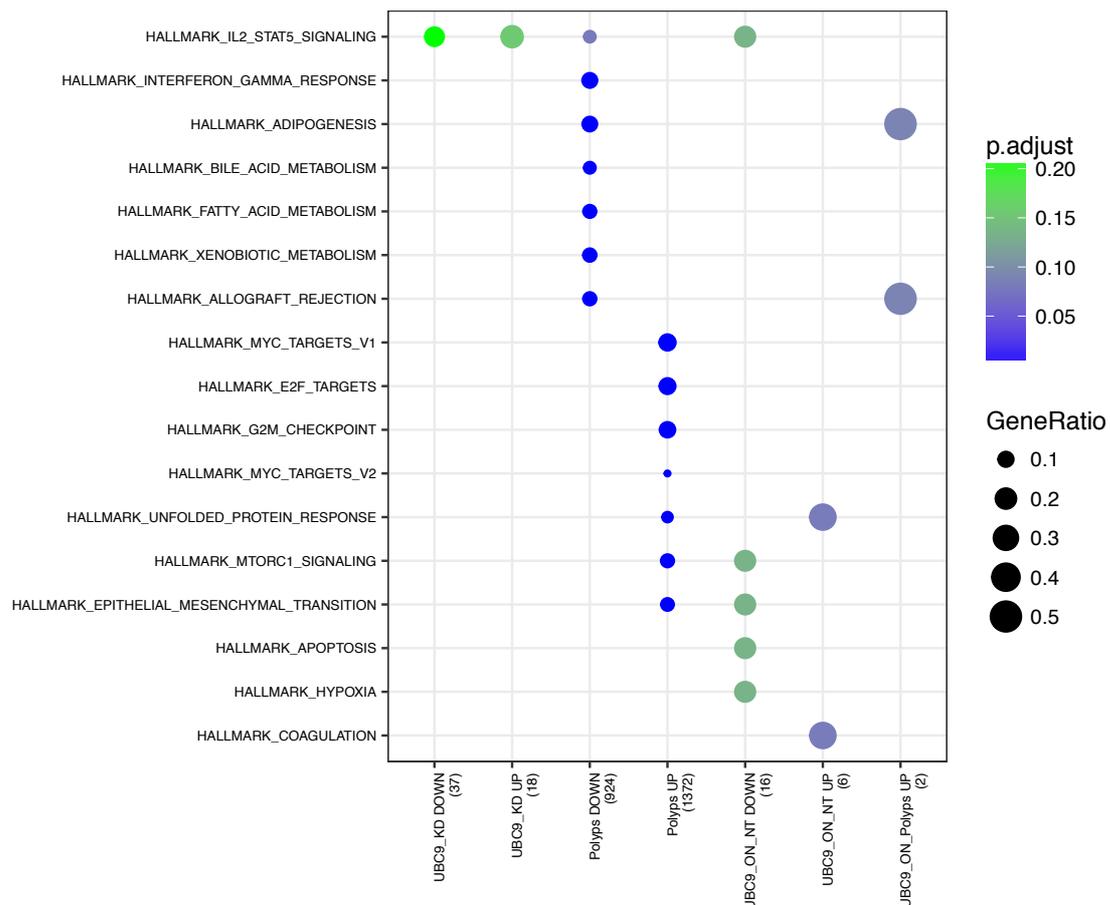
Indeed, when looking at the type of the tissue, out of the 26595 annotated genes, the analysis revealed 11647 differentially expressed, 5777 downregulated and 5870 upregulated in polyps when compared to normal tissue (Figure 46A), with a clear differential pattern of gene expression where two major clusters of probes could be identified (Figure 46C). After a Gene Set Enrichment Analysis (GSEA), 335 pathways showed statistically significant differential expression within this comparison, 48 pathways downregulated and 287 pathways enriched in polyps, amongst which, as expected, a lot of cancer pathways such as prostate cancer, lung cancer, colorectal cancer, endometrial cancer, pancreatic cancer, thyroid cancer and typical cancer pathways such as Myc etc.

On the contrary, when looking at the genotype, even though 272 genes are differentially expressed, of which 174 downregulated and 98 upregulated in *Ubc9*<sup>+/-</sup> when compared to *Ubc9*<sup>+/+</sup> samples (Figure 46B), no pathways appeared enriched or depleted after the GSEA within this comparison. This suggests that the knockdown of *Ubc9* doesn't have a clear effect, which is further confirmed by the pattern of gene expression, where no clear clusters of probes could be identified between *Ubc9*<sup>+/-</sup> and *Ubc9*<sup>+/+</sup> samples (Figure 46D).



**Figure 46 Transcriptomic analysis of samples collected after APC loss. A:** Volcano plot representing the overall summary between normal tissue and polyps. Thresholds: log2 fold change and significant difference at q-value<0.1. x-axis: log2 fold change, y-axis: q-value. **B:** Volcano plot representing the overall summary between *Ubc9*<sup>+/+</sup> (WT) and *Ubc9*<sup>+/-</sup> (KD). Thresholds: log2 fold change and significant difference at q-value<0.1. x-axis: log2 fold change, y-axis: q-value. **C:** Effect of polyps. Heatmap illustrating hierarchical clustering analysis of the probe sets differentially expressed in polyps when compared to normal tissue. The relative expression for each individual at a given probe was reflected by its color intensity. In the heat map, yellow represents overexpressed genes, and blue represents underexpressed genes. **D:** Effect on *Ubc9* haploinsufficiency. Heatmap illustrating hierarchical clustering analysis of the probe sets differentially expressed in *Ubc9*<sup>+/-</sup> (WT) when compared to *Ubc9*<sup>+/+</sup> (Heterozygous). The relative expression for each individual at a given probe was reflected by its color intensity. In the heat map, yellow represents overexpressed genes, and blue represents underexpressed genes.

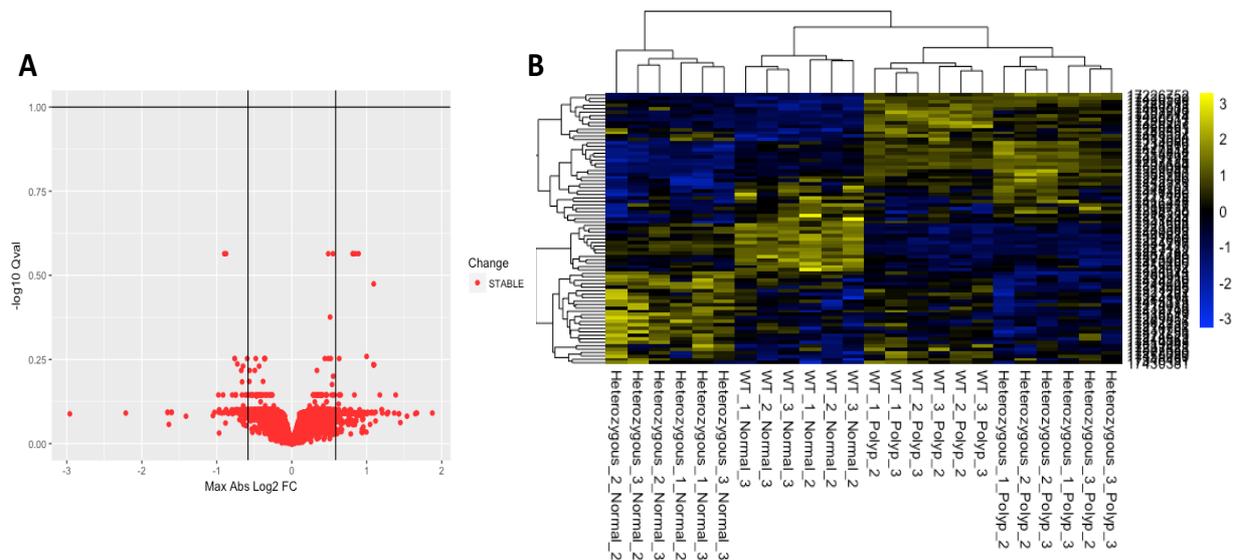
When we performed over-representation analysis, for the differentially expressed gene list of each dataset we confirmed that a lot of molecular signatures linked to cancer are dysregulated in polyps, such as the Myc targets and the G2M checkpoint that appear upregulated in polyps whereas no signature stands out as differentially regulated when looking at the genotype (Figure 47). This result further confirms our observation that there is an effect when looking at the tissue but the genotype doesn't seem to have any striking effect.



**Figure 47 Gene set over-representation analysis for samples collected after APC loss.** Over-representation analysis for each differentially expressed gene list considering the MSigDB hallmark molecular signature using a right-tail modified Fisher's exact test and the hypergeometric distribution to provide p-value. In order from left to right: downregulated in *Ubc9*<sup>+/-</sup>, upregulated in *Ubc9*<sup>+/-</sup>, downregulated in polyps, upregulated in polyps, downregulated in normal tissue of *Ubc9*<sup>+/-</sup>, upregulated in normal tissue of *Ubc9*<sup>+/-</sup>, upregulated in polyps *Ubc9*<sup>+/-</sup>.

When we take into account both genotype and tissue specificity and look into genes that are differentially expressed in *Ubc9*<sup>+/-</sup> polyps compared to *Ubc9*<sup>+/+</sup> polyps we cannot detect any statistically significant differentially expressed genes (Figure 48A). However, we observe different clusters of probes (Figure 48B) and some interesting pathways appear from

the GSEA, specifically enriched or depleted in *Ubc9<sup>+/-</sup>* polyps. In particular, out of these pathways we distinguish the enrichment in *Ubc9<sup>+/-</sup>* polyps of the neuroactive ligand-receptor interaction and the GPCR ligand binding (Annex 2), the largest family of cell-surface molecules involved in signal transmission, that are involved in cell signalling via G-coupled proteins receptors, ranging from hormones to WNT ligands, and are linked to cell proliferation, division, migration and subsequently cancer (Dorsam and Gutkind, 2007). Moreover, we also distinguish pathways related to transforming growth factor-beta (TGF-beta) and specifically the signaling by TGF-beta receptor complex, the transcriptional activity of SMAD2/SMAD3:SMAD4 heterodimer and the TGF-beta signalling pathway that are depleted specifically in the *Ubc9<sup>+/-</sup>* polyps (Annex 2). TGF-beta in early stages of cancer has been shown to have anti-proliferative effects, acting as a tumor suppressor, thus its downregulation would promote the cancer initiation and the appearance of polyps which is in agreement with our findings of higher number of polyps in *Ubc9<sup>+/-</sup>* mice. Indeed, the downregulation of TGF-beta receptors in human colorectal cancers has been shown to be involved in cancer development (Matsushita *et al.*, 1999).



**Figure 48 Transcriptomic analysis of samples collected after APC loss. A:** Volcano plot representing the overall summary of genes that are expressed differentially specifically in *Ubc9<sup>+/-</sup>* polyps compared to *Ubc9<sup>+/+</sup>* polyps. Thresholds: log2 fold change and significant difference at q-value<0.1. x-axis: log2 fold change, y-axis: q-value. **B:** Effect on *Ubc9* haploinsufficiency. Heatmap illustrating hierarchical clustering analysis of the probe sets differentially expressed in *Ubc9<sup>+/-</sup>* (Heterozygous) when compared to *Ubc9<sup>+/+</sup>* (WT). The relative expression for each individual at a given probe was reflected by its color intensity. In the heat map, yellow represents overexpressed genes, and blue represents underexpressed genes.

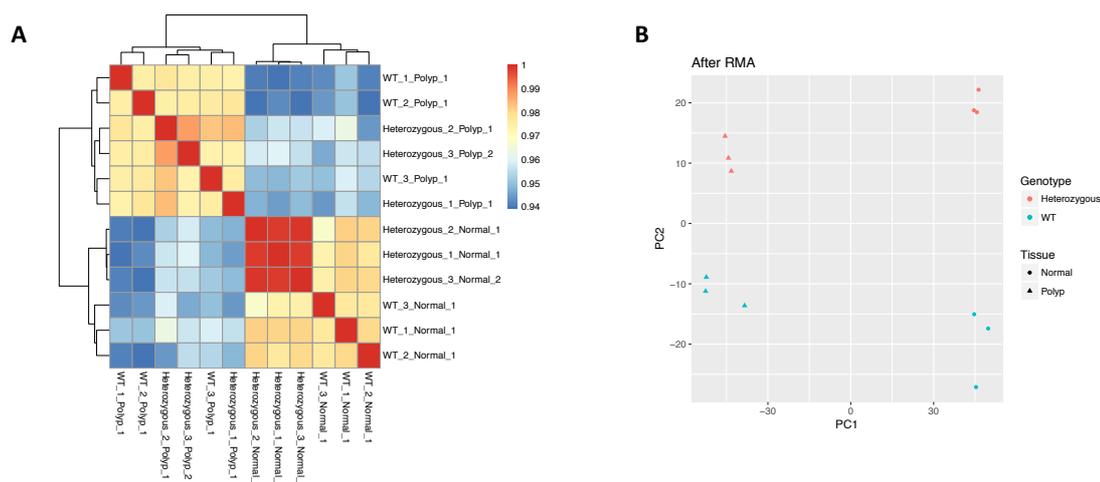
A summary of the number of genes that are upregulated and downregulated in the different comparisons is presented in the Table 6. As mentioned above, we can see a lot of genes disregulated when comparing tissue (normal vs polyp) and we see some genes disregulated when comparing the genotype (*Ubc9*<sup>+/+</sup> vs *Ubc9*<sup>+/-</sup>). However, the effect of the genotype is not striking as we can see when looking into the effect of the genotype in the tissue (Normal *Ubc9*<sup>+/+</sup> vs Normal *Ubc9*<sup>+/-</sup>, Polyps *Ubc9*<sup>+/+</sup> vs Polyps *Ubc9*<sup>+/-</sup>), where few genes appear disregulated. Lastly, no gene appears with statistically significant disregulation, when we analyze tissue and genotype specificity (Polyps *Ubc9*<sup>+/+</sup> vs Polyps *Ubc9*<sup>+/-</sup> Specific = genes specifically disregulated in polyps and *Ubc9* haploinsufficiency).

**Table 6 Summary of the comparative gene expression analysis for the samples collected after APC loss.**

	Down	Stable	Up
Ubc9 <sup>+/+</sup> vs Ubc9 <sup>+/-</sup>	174	26323	98
Normal vs Polyp	5777	14948	5870
Normal Ubc9 <sup>+/+</sup> vs Normal Ubc9 <sup>+/-</sup>	53	26498	44
Polyps Ubc9 <sup>+/+</sup> vs Polyps Ubc9 <sup>+/-</sup>	10	26566	19
Polyps Ubc9 <sup>+/+</sup> vs Polyps Ubc9 <sup>+/-</sup> Specific	0	26595	0

## Transcriptomic analysis of samples derived from AOM-DSS treatment

A sample of polyp and surrounding normal tissue per mouse was taken from three different mice. As expected the transcriptional profile is different between polyps and normal tissue, with the normal tissue of heterozygous mice clustering together with the normal tissue of the wild-type mice and, subsequently, the polyps of heterozygous and wild-type mice clustering together (Figure 49). When looking into the genotype, we observe a better correlation amongst the normal tissue of each genotype. As is clearly shown in the Figure 49A there is an increased similarity in the case of heterozygous normal samples. We do not observe the same clear distinction between genotypes in the case of polyps which would suggest that the heterozygosity of *Ubc9* has a strong effect particularly in the context of normal tissue after treatment with AOM-DSS (Figure 49).



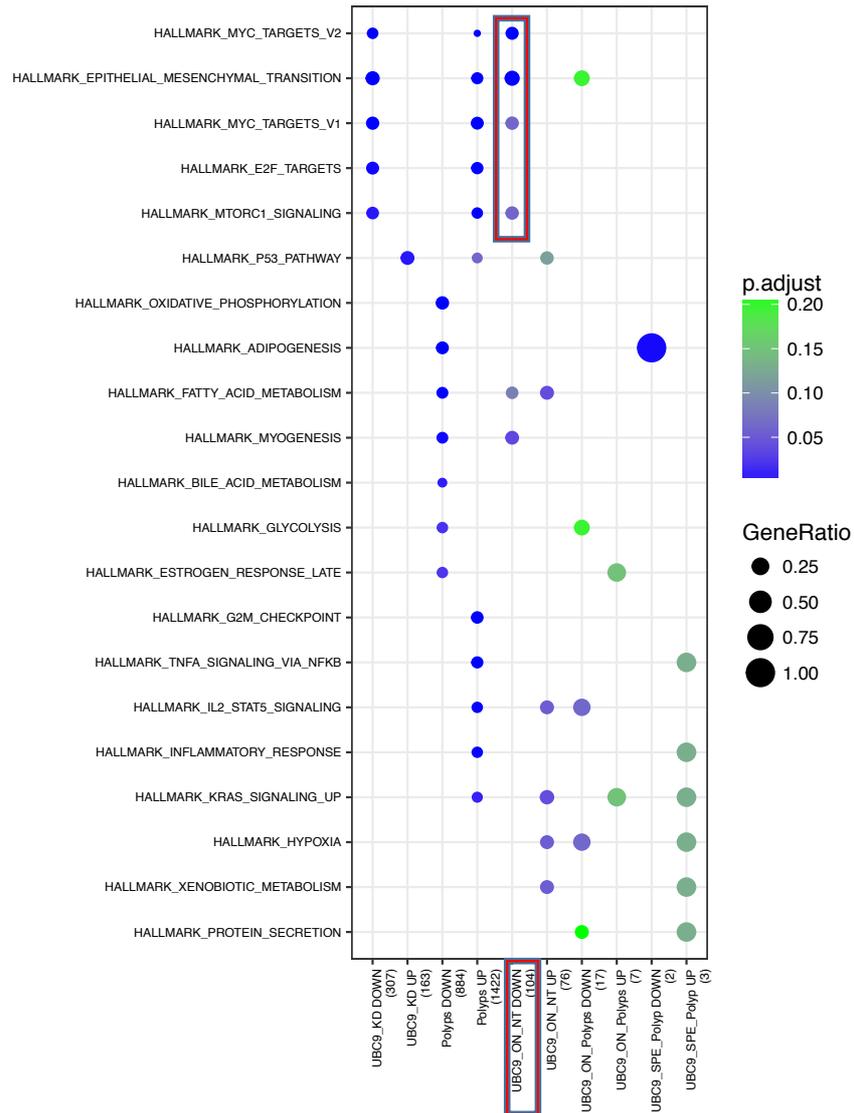
**Figure 49 Clustering after normalization of data for samples collected after AOM-DSS treatment. A:** Heatmap after RMA using Ward's distance. In the Figure we have first the genotype heterozygous or wild-type (WT) then the number of the mouse, 1,2 or 3, then the tissue normal or polyp. **B:** PCA analysis after RMA. WT: *Ucc9*<sup>+/+</sup>, Heterozygous: *Ubc9*<sup>+/-</sup>

When looking at the type of the tissue, out of the 27216 annotated genes, the analysis revealed 12618 differentially expressed, 6933 downregulated and 5685 upregulated in polyps when compared to normal tissue (Figure 50A), with a clear differential pattern of gene expression where two major clusters of probes could be identified (Figure 50C). After a Gene Set Enrichment Analysis (GSEA), 422 pathways showed statistically significant differential expression within this comparison, 47 pathways downregulated and 375 pathways enriched in



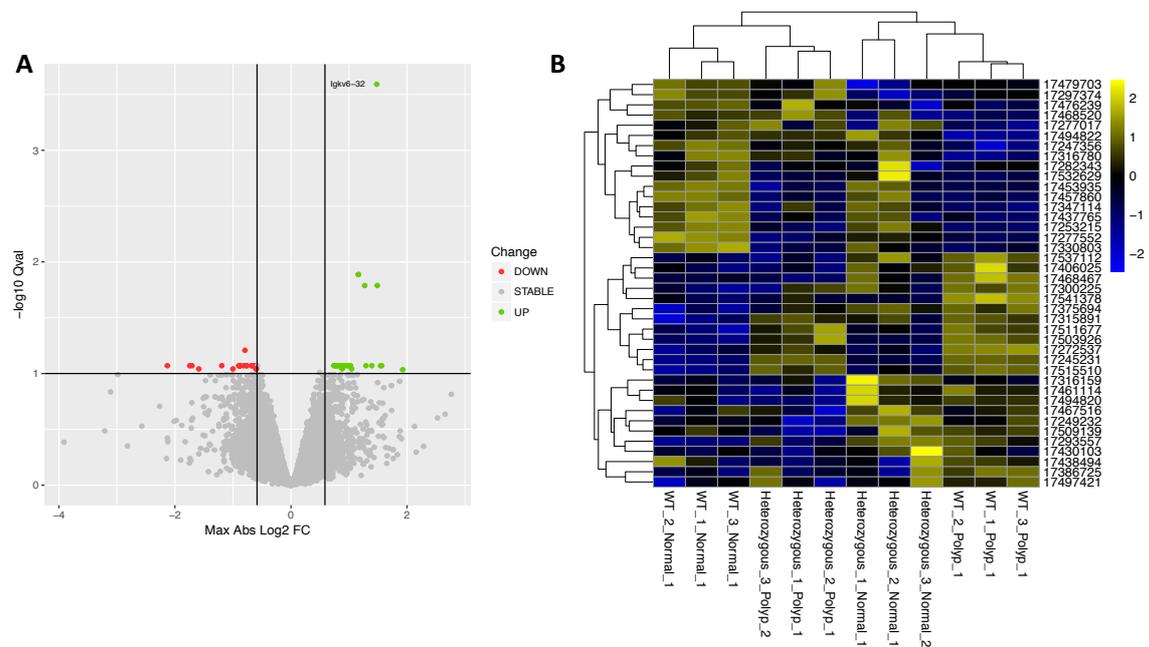
illustrating hierarchical clustering analysis of the probe sets differentially expressed in polyps when compared to normal tissue. The relative expression for each individual at a given probe was reflected by its color intensity. In the heat map, yellow represents overexpressed genes, and blue represents underexpressed genes. **D:** Effect on *Ubc9* haploinsufficiency. Heatmap illustrating hierarchical clustering analysis of the probe sets differentially expressed in *Ubc9*<sup>+/-</sup>(WT) when compared to *Ubc9*<sup>+/+</sup> (Heterozygous). The relative expression for each individual at a given probe was reflected by its color intensity. In the heat map, yellow represents overexpressed genes, and blue represents underexpressed genes.

When we performed over-representation analysis in the context of polyps, we observe as expected an upregulation of typical molecular hallmarks for cancer. Most of these cancer related hallmarks appear to be downregulated at *Ubc9*<sup>+/-</sup> only in the context of normal tissue. More specifically we have a downregulation of typical molecular hallmarks related to cancer such as Myc targets, epithelial mesenchymal transition, MTPRC1 responsible for promoting cell growth and proliferation (Figure 51). Simultaneously, we observe an upregulation of the anti-inflammatory cytokine IL-2 in the normal tissue of *Ubc9*<sup>+/-</sup> mice. Interestingly, in the *Ubc9*<sup>+/-</sup> we observe upregulation of the p53 pathway suggesting an upregulation of repair mechanisms (Figure 51). Therefore, it would seem that in the context of AOM-DSS induced carcinogenesis, in the *Ubc9*<sup>+/-</sup> mice there is a downregulation of cancer signatures and an increased damage response.



**Figure 51 Gene set over-representation analysis.** Over-representation analysis for each differentially expressed gene list considering the MSigDB hallmark molecular signature using a right-tail modified Fisher's exact test and the hypergeometric distribution to provide p-value. In order from left to right: downregulated in Ubc9<sup>+/-</sup>, upregulated in Ubc9<sup>+/-</sup>, downregulated in polyps, upregulated in polyps, downregulated in normal tissue of Ubc9<sup>+/-</sup>, upregulated in normal tissue of Ubc9<sup>+/-</sup>, downregulated in polyps Ubc9<sup>+/-</sup>, upregulated in polyps Ubc9<sup>+/+</sup>, downregulated specifically in polyps Ubc9<sup>+/-</sup>, upregulated specifically in polyps Ubc9<sup>+/+</sup> (specifically= statistically important in the particular combination of tissue and genotype).

When we take into account both genotype and tissue specificity and look into genes that are differentially expressed in *Ubc9*<sup>+/-</sup> polyps compared to *Ubc9*<sup>+/+</sup> polyps we detect 47 statistically significant differentially expressed genes (Figure 52A). Additionally, multiple clusters of probes can be seen (Figure 52B) and some interesting pathways appear from the GSEA, specifically enriched or depleted in *Ubc9*<sup>+/-</sup> polyps. Out of these pathways we distinguish the downregulation of pathways related to cytokine cytokine receptor interaction, chemokine receptors bind chemokines, cytokine signaling in immune system and the downregulation of other pro-inflammatory pathways like IL1R pathway, interferon alpha-beta signaling, signaling by ILS and interferon signaling (Annex 3). Finally, we also observe the downregulation of cancer pathways and more specifically, bladder cancer pancreatic cancer and pathways in cancer in general (Annex 3).



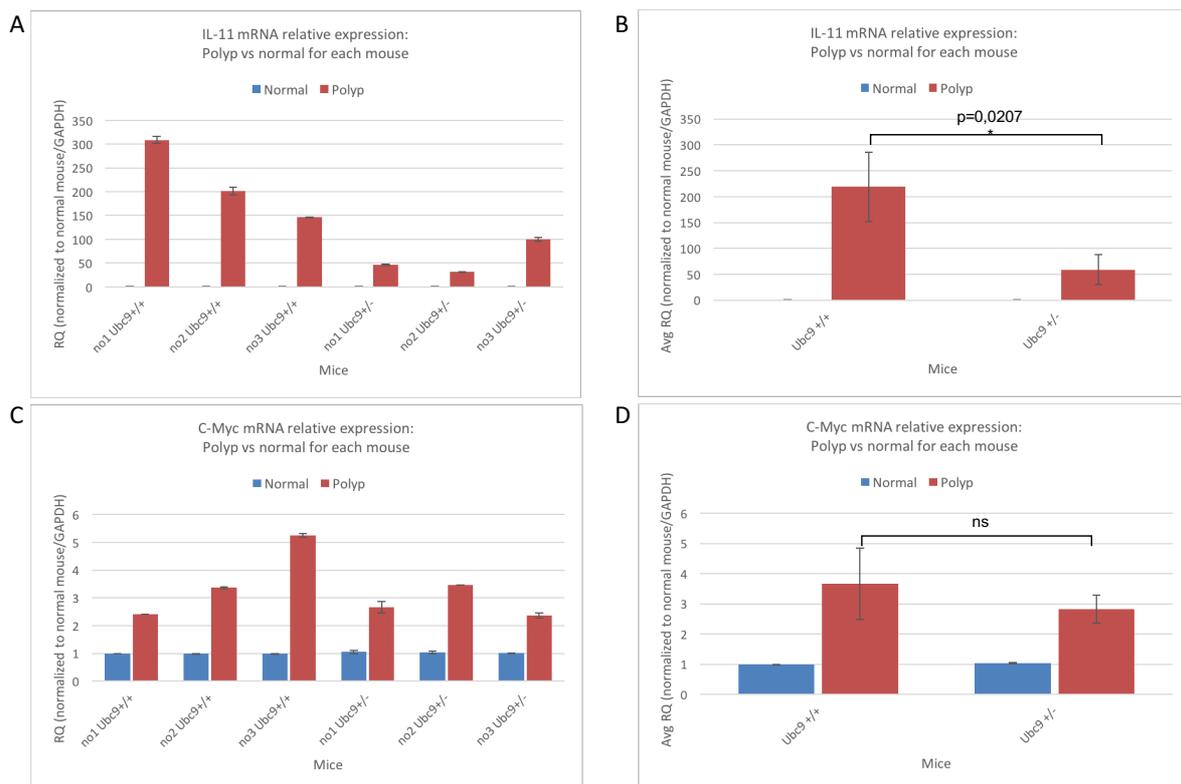
**Figure 52 Transcriptomic analysis of samples collected after AOM-DSS treatment.**

**A:** Volcano plot representing the overall summary of genes that are differentially expressed specifically in *Ubc9*<sup>+/-</sup> polyps compared to *Ubc9*<sup>+/+</sup> polyps. Thresholds: log2 fold change and significant difference at q-value<0.1. x-axis: log2 fold change, y-axis: q-value. **B:** Effect on *Ubc9* haploinsufficiency. Heatmap illustrating hierarchical clustering analysis of the probe sets differentially expressed in *Ubc9*<sup>+/-</sup> (Heterozygous) when compared to *Ubc9*<sup>+/+</sup> (WT). The relative expression for each individual at a given probe was reflected by its color intensity. In the heat map, yellow represents overexpressed genes, and blue represents underexpressed genes.

Amongst the pro-inflammatory genes that appear downregulated in *Ubc9*<sup>+/-</sup> polyps when compared to *Ubc9*<sup>+/+</sup> polyps we distinguish IL-6ra with 1,7-fold change and IL-11 with 2,9-fold change less in *Ubc9*<sup>+/-</sup> polyps. IL-6 in an inflammatory cytokine that is required for the survival of the intestinal epithelial cells and the initiation and development of colitis-

associated cancer (Grivennikov *et al.*, 2009). IL-11 is a member of the IL-6 family promoting colitis-associated tumorigenesis and progression (Putoczki *et al.*, 2013). These results suggest that reduction of UBC9 could reduce inflammation, which may contribute at least partially to the anticancer effect in AOM/DSS-induced colon tumorigenesis.

The possible suppressive effect of the reduction of UBC9 on the inflammatory mediator IL-11 was assessed by qPCR in samples from the three different mice treated with AOM-DSS. Firstly we observed a major upregulation of IL-11 in all polyp tissues comparing to the normal tissue of each mouse (Figure 53A, 53B). Secondly, we have a remarkable downregulation of IL-11 in each *Ubc9*<sup>+/-</sup> mouse comparing to the wild-type ones (Figure 53A) with the IL-11 getting reduced by 77.7% in *Ubc9*<sup>+/-</sup> mouse (Figure 53B). C-Myc was used as control where we can observe the upregulation of c-Myc in the polyps of both genotypes comparing to the normal tissue whereas between the genotypes we observe a downregulation in the case of *Ubc9*<sup>+/-</sup> mice which is not statistically significant.



**Figure 53 Downregulation of IL-11 in *Ubc9*<sup>+/-</sup> mice after AOM-DSS treatment.**

**A, B:** Relative expression level of IL-11 mRNA in polyp and normal tissue of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> mice treated with AOM-DSS was analyzed by qPCR. Mean  $\pm$ SD, n=3, p=0.0207.  
**C, D:** Relative expression level of c-Myc mRNA in polyp and normal tissue of *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>+/-</sup> mice treated with AOM-DSS was analyzed by qPCR. Mean  $\pm$ SD, n=3, p=0.3993.

A summary of the number of genes that are significantly upregulated and downregulated in the different comparisons is presented in the Table 7. As mentioned above, we can see a lot of genes disregulated when comparing tissue (normal vs polyp) and when comparing the genotype (*Ubc9*<sup>+/+</sup> vs *Ubc9*<sup>+/-</sup>). The effect of the genotype seems important for both normal and polyp tissue (Normal *Ubc9*<sup>+/+</sup> vs Normal *Ubc9*<sup>+/-</sup>, Polyps *Ubc9*<sup>+/+</sup> vs Polyps *Ubc9*<sup>+/-</sup>), with normal tissue presenting more disregulated genes. Furthermore, statistically significant disregulation of a number of genes appears when we are analyzing tissue and genotype specificity (Polyps *Ubc9*<sup>+/+</sup> vs Polyps *Ubc9*<sup>+/-</sup> Specific = genes specifically disregulated in polyps and *Ubc9* haploinsufficiency).

**Table 7 Summary of the comparative gene expression analysis for the samples collected after AOM-DSS treatment.** Threshold: 1,5 fold change, adjusted p-value<0.1.

	Down	Stable	Up
Ubc9 <sup>+/+</sup> vs Ubc9 <sup>+/-</sup>	1200	25078	938
Normal vs Polyp	6933	14598	5685
Normal Ubc9 <sup>+/+</sup> vs Normal Ubc9 <sup>+/-</sup>	392	26480	344
Polyps Ubc9 <sup>+/+</sup> vs Polyps Ubc9 <sup>+/-</sup>	69	27085	62
Polyps Ubc9 <sup>+/+</sup> vs Polyps Ubc9 <sup>+/-</sup> Specific	25	27169	22

# **DISCUSSION**

## Introduction

Since its discovery around twenty years ago (Matunis, Coutavas and Blobel, 1996; Psakhye and Jentsch, 2012), post-translational modification by the Small Ubiquitin-related MOdifier (SUMO) has emerged as an essential regulatory mechanism of protein function affecting the interactions of its substrates with their protein and nucleic acid partners, their localization, stability or enzymatic activity (Hay, 2005; Seeler *et al.*, 2007; Hochstrasser, 2009; Flotho and Melchior, 2013). The number of known SUMO substrates and the processes in which SUMOylation is involved keeps increasing over the years. Moreover, accumulating evidence point to a complexity of the impact of SUMOylation in biological processes with many studies presenting controversial results. The existence of three SUMO isoforms, their capacity to form polymers, and the ability to target whole protein complexes rather than individual substrates for phenotype determination (Psakhye and Jentsch, 2012) provide for a large spectrum of combinations resulting in the pleiotropic effects of this modification.

Sumoylation relies on a fine equilibrium between conjugating and deconjugating activities and it is likely that perturbation in this balance contributes to healthy and disease processes. While information on the specific roles of the different E3 ligases and SENP enzymes is accumulating, our knowledge of the biological outputs of global changes of SUMOylation and more specifically the consequences of the distortion of sumoylation/desumoylation equilibrium still remains highly fragmentary (Liu and Shuai, 2008). In this study we focused on the consequences of global SUMOylation decrease on cellular transformation and colorectal oncogenesis. To that purpose, we used our unique mouse models for partial ( $Ubc9^{+/-}$ ) or conditional ( $Ubc9^{fl/-}$ ) inactivation of *Ubc9* permitting us to study the role of global sumoylation at the cell, tissue and whole organism levels.

## SUMO and cellular proliferation

We investigated our first goal regarding the impact of the decrease of global SUMOylation in cells using primary and transformed MEFs.

The data collected in this *in vitro* part of the project reveal that primary cells are able to survive the loss of SUMOylation. In particular, we see that half dose of UBC9 does not alter the cell growth, whereas the complete loss of UBC9 leads to decrease in the proliferation of primary MEFs. Importantly, after analysis of the cell cycle and the apoptosis profile we see that the cells do not arrest in any specific cell cycle phase after 4-OHT treatment and that the majority of the cells do not undergo detectable apoptosis. Previous studies in our lab demonstrated that UBC9 depletion leads to a senescence-like growth arrest in primary human fibroblasts (Neyret-Kahn *et al.*, 2013). Therefore, it is likely that the growth arrest observed in the growth curves is due to senescence-like growth arrest. That would also explain the higher amount of cells in G0-G1 phase observed in primary cells and the high percentage of cells detected as live in the analysis of the apoptosis as the Annexin V assay is not able to distinguish between proliferating and senescent cells.

On the other hand, we see that the transformed cells show an increased sensibility to the loss of *Ubc9*. In particular, in only four days after loss of *Ubc9* we have a high mortality due to apoptosis, which increases after seven days leading to death of the transformed cells. Already two days after *Ubc9* knockout, we observe an accumulation of cells in the G2-M phase, which increases after 7 days leading to a decrease of the cell population in G0-G1 phase. These results, together with the observation of a striking percentage of polyploidy after 7 days of *Ubc9* knockout suggest that loss of SUMOylation leads to a mitotic defect impaired cytokinesis. The importance of SUMOylation for the mitosis has been shown by different studies. The first time that problems in mitosis were described after SUMOylation deficiency was in yeast, where mitotic problems were observed at a mutant for *Hus5*, a homologue of *Ubc9* (Al-Khodairy *et al.*, 1995). Since then, mitotic defects due to SUMOylation deficiency were described in most of the organisms studied. Our lab has demonstrated that the embryonic lethality in *Ubc9*<sup>-/-</sup> mice is due to problems in mitosis (Nacerddine *et al.*, 2005). From a mechanistic point of view, during mitosis, SUMO is concentrated in the kinetochore where it modifies numerous SUMO substrates and coordinates multiple centromere and kinetochore activities leading to the control of chromosome segregation by SUMOylation

(Wan, Subramonian and Zhang, 2012). Consequently, the results observed here are in accordance with the crucial role of SUMOylation in mitosis.

The importance of SUMOylation for the survival of cancer cells has been suggested from other studies. For example, it has been shown that inhibition of SUMOylation by genetic means specifically disables Myc-induced proliferation, through G2/M cell-cycle arrest, polyploidy, and apoptosis as in our case (Hoellein *et al.*, 2014). The same phenotype was also reported by another study after sh-RNA for SAE2 and UBC9 in human cancer cells (He *et al.*, 2015). Taken all these into account, and since our results were consistent in both p53DN/HRasV12 and SV40T/HRasV12 transformed MEFs, we propose that the drastic mortality of the transformed cells deficient for SUMOylation when compared to primary cells is due to their characteristics as transformed cells, and not linked to a particular oncogene. This strengthens the consideration of SUMOylation as a “non-oncogene addiction” (Kessler *et al.*, 2012), making transformed cells more sensitive to severe alterations on the SUMOylation balance. Since for the development of treatments against cancer there must be a suitable therapeutic window, where there is activity toward the tumor cell but not normal cells this non-oncogene addition could be exploited therapeutically. Therefore, from a therapeutic standpoint, in our case, the difference in sensibility of the loss of SUMOylation between the primary and transformed cells opens new therapeutic opportunities, in which, inhibition of SUMOylation could have deleterious effects in cancer while the healthy cells will be able to tolerate such modification of the UBC9 levels.

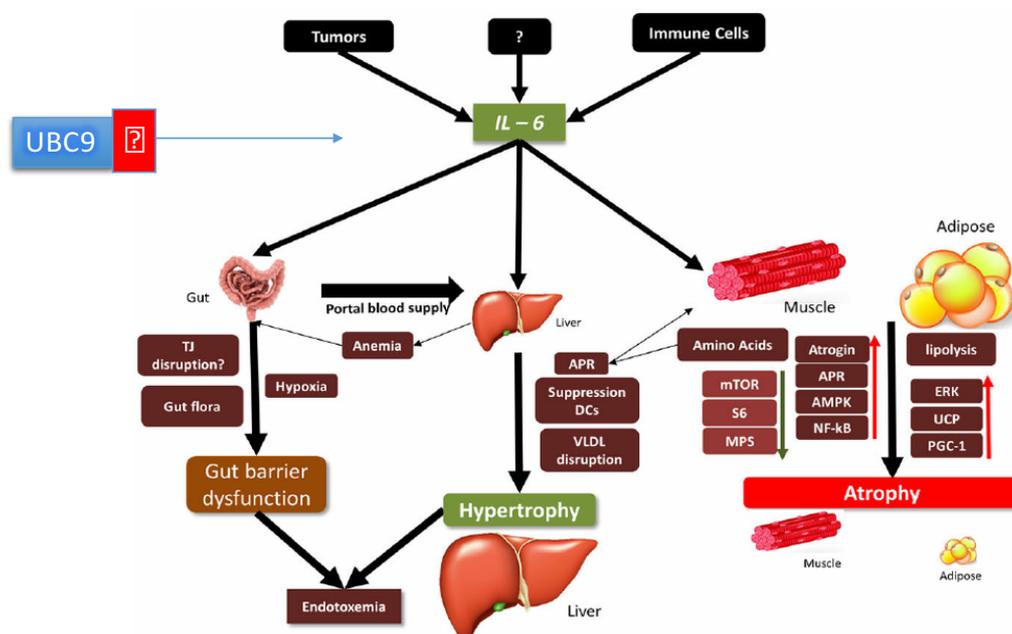
Surprisingly, although the complete loss of UBC9 leads to death of the transformed cells, paradoxically, the reduction of UBC9 to 50% leads to increased proliferation of the transformed MEFs without affecting the proliferation of primary MEFs. The transformation capacity of the cells was assessed by the use of three *ex vivo* tests. In all of the assays, MEFs haploinsufficient for *Ubc9* showed a higher transformation capacity. SUMOylation is mostly associated with transcriptional repression, with few studies showing a role for SUMO also in transcriptional activation (Rosonina, Duncan and Manley, 2010). Indeed, a study positioned SUMO at the promoters of the transcriptionally active genes but not repressed genes, while, unexpectedly, the impairment of *Ubc9* function resulted in increased transcription of these genes. It would seem that reducing SUMOylation at an induced promoter impedes the ability of the cell to shut off its transcription (Rosonina, Duncan and Manley, 2010).

It is apparent that modulating the levels of SUMOylation could have either promoting or suppressive effects. Our results prove that the disturbance of the equilibrium of SUMOylation leads to different outcomes depending on the level of SUMOylation decrease. Experiments that we are currently performing further confirm those results. Using SUMO inhibitors we observe controversial results depending on the dose used, and thus the inhibition of the pathway. In particular, upon a certain level of inhibition of the pathway, the transformed cells increase their proliferation whereas in higher levels of inhibition they cannot survive the loss of SUMOylation (data not shown). In line with this notion, recent results in our lab suggest that different levels of *Ubc9* impairment with complete knockout of *Ubc9* results in arrest of reprogramming whereas knockdown of *Ubc9* to half amount clearly increases the reprogramming (data not published). Together all these data, highlight that despite the therapeutic potential of targeting global SUMOylation, it would be important to take into account and further investigate the importance of a dose-dependent approach in the use of the SUMO pathway for potential future therapies.

## **Mouse xenografts**

To assess the transformation capacity *in vivo*, we performed xenograft test in nude mice. In agreement with our *in vitro* results, we observe a remarkably faster growth of the *Ubc9*<sup>+/-</sup> transformed cells when compared to the wild-type ones. However, the knockout of *Ubc9* in this model, although slightly slowed down, was not sufficient to impair the tumor growth. This could be due to the small percentage of transformed cells that do not apoptose after loss of *Ubc9*. Surprisingly, the *Ubc9*<sup>+/-</sup> transformed cells appeared to cause a severe phenotype of cachexia to the nude mice, with massive loss of body mass resulting in their death, condition that was not observed in *Ubc9* KO, nor in *Ubc9*<sup>+/+</sup> cells. Cancer cachexia is an important condition that appears at the final stages of 80% of cancer cases and is responsible for the death in 22% of the cases (Aoyagi *et al.*, 2015). It is a complex metabolic syndrome that occurs as physiological response to inflammation where numerous cytokines, including TNF- $\alpha$ , IL-1, IL-6, and interferon-gamma (IFN- $\gamma$ ), have been associated with the etiology of this condition (Carson and Baltgalvis, 2010; Fearon, Glass and Guttridge, 2012; Narsale and Carson, 2014). Particularly, the critical role of IL-6 has been demonstrated and is illustrated in the Figure 54. Previous studies in the lab have shown that *Ubc9* KO of mouse bone marrow-derived dendritic cells secreted higher amount of pro-inflammatory cytokines such as IL-6 and TNF- $\alpha$  when compared to wildtype ones (Decque *et al.*, 2016) suggesting

that downregulation of SUMOylation may lead to higher production of those pro-inflammatory cytokines. Tamoxifen has been used in the hormonal treatment of breast cancer and has a complex tissue-dependent mode of action. In breast cancer, it has been associated with changes in the production of a number of cytokines, including insulin-like growth factor (IGF), TGF- $\beta$ , IL-6 (McNamara *et al.*, 2001; Paduch and Kandefers-Szerszen, 2005). Based on our results, our hypothesis is that the *Ubc9<sup>fl/-</sup>* tumors induce the production of higher amount of IL-6 and TNF- $\alpha$  when compared to the *Ubc9<sup>+/+</sup>* ones resulting in cancer cachexia. After tamoxifen treatment and inactivation of the floxed allele, at the resulting *Ubc9* KO tumors, we have the decrease of the levels of IL-6 and TNF- $\alpha$ , preventing cancer cachexia. In order to evaluate this hypothesis, more experiments have to be performed in xenografts derived from *Ubc9<sup>+/-</sup>*, *Ubc9<sup>fl/-</sup>* and *Ubc9<sup>+/+</sup>* cells with tamoxifen treatment or not while measuring the production of cytokines in the mouse. This should also be performed at different time points during cell culture to evaluate whether the possible effect in cytokine production would be the same or requires the use of the mouse host system. Nevertheless, the elucidation of the role of *Ubc9* in cancer cachexia could be promising for the development of treatments which would protect cancer patients of this condition and could possibly reveal new mechanism of IL-6 regulation.

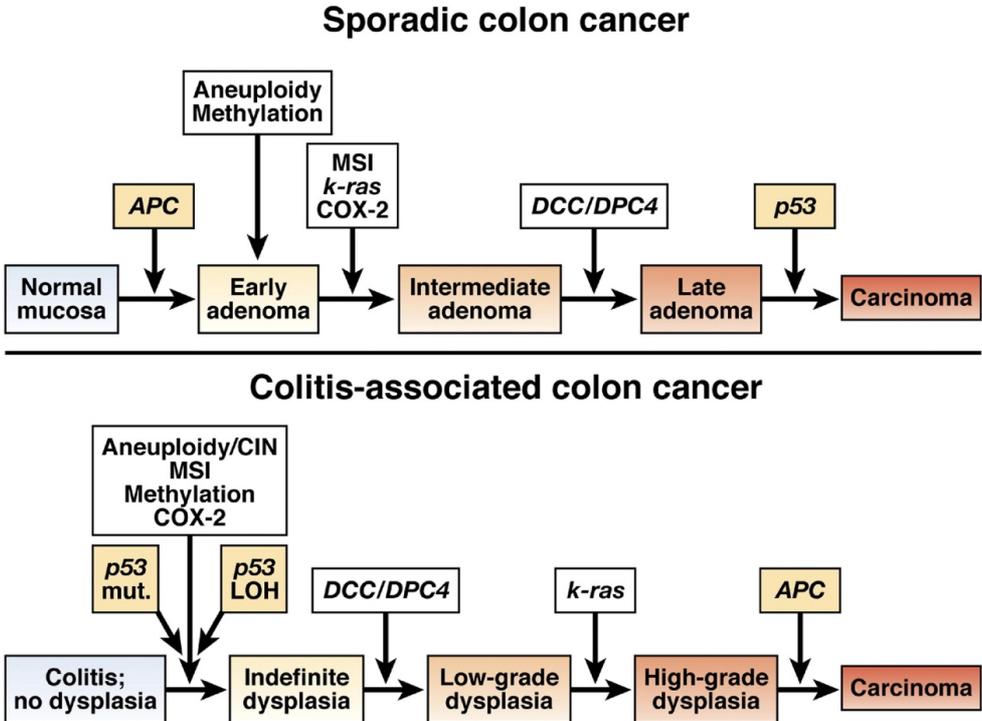


**Figure 54 Representative figure illustrating the systemic effect of IL-6 dependent cachexia in vivo.** Tumor and immune cells, such as macrophages, promote the production of IL-6 that affects various tissues such as gut, liver, muscle and adipose tissue. Inflammatory and degradation pathways are expressed in muscle and liver leading to muscle atrophy and liver dysfunction. Adipose tissue undergoes rapid lipolysis, thus atrophy and the permeability of the gut is increased due to disruption in tight junction proteins and anemia. We propose that UBC9 may regulate the production of IL-6 (modified from Narsale and Carson, 2014).

### Ubc9 haploinsufficiency in colorectal carcinogenesis

In the second part of the thesis, we focused on the role of the reduction of SUMOylation in colorectal cancer, using our unique mouse models, haploinsufficient for *Ubc9*. The effect of UBC9 knockdown was evaluated in different models for induction of colorectal oncogenesis, the sporadic cancer model dependent on the loss of APC (genetic model), and the colitis-associated cancer model dependent on an alkylating agent with an inflammatory agent (AOM-DSS: chemical model).

In the case of the chemical model, the AOM (azoxymethane) alone was not sufficient to produce any significant phenotype. However, when AOM was combined with the inflammatory agent DSS, we observed an important decrease of the tumorigenesis in the *Ubc9* haploinsufficient mice. On the other hand, in the genetic model, after loss of APC, *Ubc9* haploinsufficient mice display enhanced intestinal tumorigenesis. Those seemingly contradictory results could be explained by the differences of the two colorectal models (Figure 55). Nevertheless, in both cases, although the number of polyps and tumor burden is different, no difference is observed in the size of polyps suggesting that the effect of *Ubc9* is on the initiation rather than the progression of colorectal cancer.



**Figure 55** Differences between sporadic and colitis-associated colorectal cancer (Ullman and Itzkowitz, 2011).

The two models share certain genetic features, such as chromosomal and microsatellite instability and hypermethylation. The majority of the molecular dysregulation occurring in sporadic colorectal carcinogenesis appears in colitis-associated adenocarcinoma too, but differences have been observed in both their timing and prevalence. In the sporadic colon cancer, the loss of APC gene takes place early and a p53 mutation occurs in later stages whereas in the colitis-associated colon cancer APC and p53 mutations occur in reverse manner with p53 mutations playing pivotal role in the cancer development. In the sporadic model, p53 mutations are observed in aggressive lesions whereas in the colitis-associated model p53 mutations are seen within non-dysplastic mucosa.

### **Role of Ubc9 haploinsufficiency in genetically induced tumorigenesis**

The sporadic model presents higher prevalence of k-ras mutations and targets the canonical Wnt signaling. The  $\beta$ -catenin or canonical Wnt pathway is a major regulator of stem/progenitor cell maintenance, expansion, and lineage specification in both embryonic and adult tissues. After loss of APC in the sporadic model, activation of this pathway affects the Lgr5<sup>+</sup> stem cells that, in such case, have been proven to be the cells of origin of colorectal cancer. Loss of APC specifically from colonic stem cells results in progressively growing neoplasms, whereas its loss from transit-amplifying cells (located near the crypt base) does not. When we studied the effect of UBC9 in normal stem cells by the generation and use of the *Ubc9*<sup>+/+</sup>/Lgr5-EGFP-IRES-CreERT2 and *Ubc9*<sup>+/-</sup>/Lgr5-EGFP-IRES-CreERT2 mouse model, we had an unexpected result: remarkably, the *Ubc9* haploinsufficiency leads to an increased number of Lgr5<sup>+</sup> stem cells in the normal intestinal crypts. The increased number of stem cells that we observe in the entire intestine means an increased number of potential tumor-initiating cells, which could explain the increased tumorigenesis observed in the *Ubc9*<sup>+/-</sup> mice induced by APC. Previous studies in the lab have shown that acute loss of sumoylation in adult primarily affects the small intestine, and in first instance the stem cell compartment, leading to rapid death (Demarque *et al.*, 2011) which confirms a possible role of SUMOylation in the stem cells that once again seems dose-dependent. In line with this notion, *Ubc9* haploinsufficient cells demonstrate strong increase in reprogramming as it has been recently demonstrated by unpublished studies in our lab, strongly confirm the notion that

Ubc9 dysregulation has an effect in the stemness of the cell. Further experiments are currently being performed to determine the transcriptional profile of FACS-isolated Lgr5-GFP<sup>high</sup> cells in *Ubc9*<sup>+/-</sup> mice for comparison with Lgr5-GFP<sup>high</sup> cells from *Ubc9*<sup>+/+</sup> mice. These results might help us to elucidate a transcriptional basis to the effect of *Ubc9* haploinsufficiency on the crypt stem cells.

To better understand the effect of *Ubc9* haploinsufficiency on these processes, we performed a transcriptomic analysis through which we found the depletion of pathways involved in the TGF-beta signaling in *Ubc9*<sup>+/-</sup> polyps. TGF-beta in early stages of cancer has been shown to have anti-proliferative effects, acting as a tumor suppressor, thus its downregulation would promote the cancer initiation and the appearance of polyps which is in agreement with our findings of higher number of polyps in *Ubc9*<sup>+/-</sup> mice. Indeed, the downregulation of TGF-beta receptors in human colorectal cancers has been shown to be involved in cancer development (Matsushita *et al.*, 1999). Moreover, we have the upregulation of pathways involved in cell signalling via G-coupled proteins receptors, that are linked to cell proliferation, division, migration and subsequently cancer (Dorsam and Gutkind, 2007).

# Role of Ubc9 haploinsufficiency in chemically induced tumorigenesis

The molecular pathogenesis of the colitis-associated cancer remains incompletely understood as a lot of pathways are affected (Figure 56). In our case we used a two-step model that is comprised of a chemical induction of DNA damage followed by repeated cycles of colitis. In contrast to APC loss, chronic inflammation and other conditions that increase NF-κB signaling support the notion that a dedifferentiation step can occur in intestinal epithelia, leading to the development of colorectal cancer by a “top down” approach (Schwitalla *et al.*, 2013). The colitis-associated tumorigenesis is comprised of the production of pro-inflammatory cytokines, followed by induction of mutations in oncogenes and tumor suppressor genes, mostly p53, and subsequently the production of ROS. Inflammation creates an environment that favors the activation of proliferation and anti-apoptotic properties of premalignant epithelial cells thus promoting tumor initiation and subsequently, the accumulation of mutations in the hyper-proliferative cells leads to the tumor progression and metastasis.

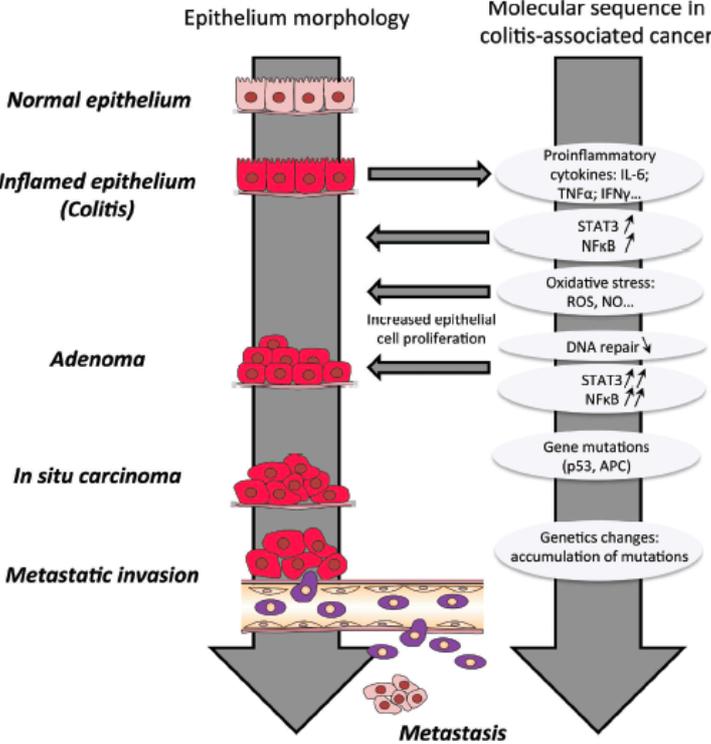


Figure 56 Molecular sequence in colitis-associated cancer (Viennois, Chen and Merlin, 2013).

The transcriptomic analysis of samples derived from AOM-DSS induced tumorigenesis revealed a potential different intestinal environment upon inflammation that does not favor the inflammation-induced cancer in the *Ubc9<sup>+/-</sup>* mice. When mice are treated with AOM-DSS it would seem that there is a downregulation of cancer signatures and an increased damage response in normal tissue of *Ubc9<sup>+/-</sup>* mice when compared to the wild-type ones. Moreover, in *Ubc9<sup>+/-</sup>* polyps appeared to be less inflamed as we observe a downregulation of pathways related to pro-inflammatory pathways and cytokines with an important downregulation of the inflammatory mediators IL-6ra and IL-11, that belong to the IL-6 family and have been shown to promote colitis-associated tumorigenesis and progression (Grivennikov *et al.*, 2009; Putoczki *et al.*, 2013). IL-6 and IL-11 mostly activate an overlapping set of genes in cancer, with only a few of them being more specific for IL-6 or for IL-11. A study from Putoczki *et al.* demonstrates the dominant importance of IL-11-gp130 signaling in models of colitis induced colorectal cancer affecting the STAT3 pathway. When models with the wild type gp130 are used, both IL-6 and IL-11 are required for tumor development. When models with a gp130 mutant are used, the tumorigenesis is solely dependent on IL-11. IL-11-gp130 signaling is required for enhanced cell proliferation and resistance to apoptosis. It would seem that the dysregulation of SUMOylation has an effect on inflammatory cytokines, which enhances our hypothesis of possible dysregulation of cytokines that lead to the cachectic phenotype of xenograft models due to the transformed *Ubc9<sup>fl/-</sup>* cells.

Finally, it has been shown that inflammation may also modify the intestinal microbiota prompting the overgrowth of genotoxic microorganisms, which may act as further cancer promoters. To assess any possible role of Ubc9 reduction to the microbiota composition, we are currently analyzing data that we have collected from 16S rRNA sequencing of the *Ubc9<sup>+/+</sup>* and *Ubc9<sup>+/-</sup>* mice treated with AOM-DSS or not. This analysis could shed some light into the apparently complex and multi-layered effect of altered SUMOylation balance on tumorigenicity.

**Nevertheless, in both cancer models the dysregulation of SUMOylation produced strong phenotypes linked to the initiation of colorectal cancer. We conclude that targeting SUMOylation is a promising potential tool for treatment of colorectal cancer but it is important to take into account the background of the cancer and the effect of the dose on the SUMOylation balance.**

# **MATERIALS AND METHODS**

## Cell culture

Mouse embryonic fibroblasts were obtained from embryos at day E1 $\beta$ .5 and cultured in DMEM medium (Dulbecco's Modified Eagle Medium) supplemented with 10  $\mu$ l / ml penicillin, 10  $\mu$ g / ml streptomycin and 10% fetal calf serum at 37°C, 5% CO<sub>2</sub> and 20% O<sub>2</sub>. When indicated, 100  $\mu$ g / ml of 4-hydroxy tamoxifen was added to the culture medium.

## Cell proliferation

The MEFs were seeded at  $5 \times 10^5$  cells in 75 cm<sup>2</sup> dishes. They were trypsinized and counted every 3 to 4 days, and reseeded to  $5 \times 10^5$  cells per dish. The doubling time of the population is calculated using the formula:

$$\text{Doubling time} = (\text{Log (number of day D)} - \text{Log (number seeded)}) \times 3.32$$

Alternatively, for proliferation tests over short periods, the Crystal Violet (hereafter) was used. The cells were seeded in 6-well plates at  $10^5$  cells per well, and then fixed for ten minutes in 10% formalin, pH 7, after the indicated number of days.

## Growth curves

The evaluation of the toxicity of a treatment inducing DNA damage was carried out according to the Crystal Violet method.  $10^5$  cells were seeded in 6-well plates and then treated with the genotoxic agent, done in triplicates. After 72h of culture, the cells were fixed for 10 minutes in 10% formalin pH 7, washed and then stained with 0.01% Crystal Violet solution for ten minutes at room temperature. After extensive washes, the Violet Crystal trapped in the cell membranes was solubilized with 1 ml acetic acid 10%. The relative number of cells per well was then extrapolated from the absorbance at 590 nm.

## Viral production and infection

On the first day  $4,5 \times 10^5$  Phoenix cells (HEK293T cells modified for the production of mouse tropism retroviruses) were seeded in 10 cm dishes. The following day, after changing their medium for a DMEM medium supplemented with chloroquine  $25 \mu\text{M}$ , Phoenix cells were transfected in calcium phosphate with the combination of  $10 \mu\text{g}$  of retroviral DNA,  $61 \mu\text{l}$  of  $2\text{M CaCl}_2$  diluted in  $500 \mu\text{l}$  of sterile water and  $500 \mu\text{l}$  of HBS 2X, incubated for 30 minutes at room temperature. The transfected cells were then incubated overnight at  $37^\circ\text{C}$ . On the same day  $4,5 \times 10^5$  cells-target to be infected were seeded in 10 cm dishes. The following two days, every 3 hours and for 4 times, the supernatant of the Phoenix cells was sampled, filtered through  $0.45 \mu\text{m}$ , supplemented with  $4 \mu\text{l}$  of Polybrene 1000X ( $8 \text{mg} / \text{ml}$ ) and then applied to the target cells whose medium has previously been aspirated. Finally, the antibiotic allowing the selection (puromycin  $4 \mu\text{g} / \text{ml}$  or hygromycin  $100 \mu\text{g} / \text{ml}$ ) was added and maintained until the complete mortality of the non-infected control cells was observed.

## Foci formation test

$1 \times 10^6$  cells (MEFs) were seeded in 10cm dishes. The DMEM medium was changed every 3 days. After 14 days cells were fixed with 4% PFA and then stained with Crystal Violet.

## Low density assay

200 cells were seeded in 10cm dishes. The DMEM medium was changed every 3 days. After 14 days cells were fixed with 4% PFA and then stained with Crystal Violet.

## Soft-agar

$5 \times 10^4$  cells were seeded in 6-well plates in a mix of agar and DMEM medium on top of a layer of agar.  $0.5 \text{ml}$  of DMEM medium was added on top and was changed every 3 days. After 21 days the colonies were stained with Crystal Violet.

## Cytometry

The cell cycle was analyzed using Millipore's "Cell cycle" kit based on staining with propidium iodide, according to the manufacturer's instructions. Labelled cells were analyzed on Millipore's "MUSE cell analyzer" acquiring 10,000 events.

The apoptosis profile was assessed using Millipore's "Annexin V" kit based on a Annexin V-PE and Dead Cell Marker staining, according to the manufacturer's instructions. Labelled cells were analyzed on Millipore's "MUSE Annexin V".

## Western Blot

Protein extracts from cultured cells were obtained by lysing the cells directly into Laemmli buffer containing 2% SDS, 10% glycerol, 60 mM Tris-HCl pH 6.8 and 0.01% bromophenol blue. Protein extracts from tissues were obtained by immersing the tissue in RIPA buffer (10mM Tris-HCl pH 8, 1 mM EDTA, 0.5mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 140mM NaCl, 10mM N-EthylMaleimide and protease inhibitors "Complete EDTA-free" from Roche) in "Lysing Matrix D" tubes by MP Bio. The tissues were lysed by placing the tubes 160 in MP Bio's "FastPrep24" machine, for 6 cycles of 30 seconds at maximum power, interspersed with 5 minutes of incubation on ice. The samples concentration was subsequently measured using the Bio-Rad "DC protein assay" kit, then the samples were put back in Laemmli buffer.

All the protein extracts were sonicated and incubated for 5 minutes at 95°C, before being loaded on Polyacrylamide gel for electrophoresis, and transfer onto a nitrocellulose membrane. The membrane was blocked with 5% milk, incubated with primary antibodies and then with specific secondary antibodies coupled to fluorophores allowing detection by the Odyssey infrared system.

## Antibodies

The following antibodies were used for immunoblot: anti-UBC9 rabbit monoclonal antibody (EP2938Y), anti-CTNNB1 rabbit monoclonal (D10A8), anti-CCND1 rabbit monoclonal antibody (9βGβ), anti-alpha-Tubulin mouse monoclonal antibody (B-5-1-2), anti-

SUMO-1 rabbit monoclonal antibody (Y299), anti-SUMO-2 mouse monoclonal antibody (8A2).

#### Mouse Xenografts

Suspensions of the *Ubc9<sup>+/+</sup>* or *Ubc9<sup>fl/-</sup>* p53DN-HRasV12 transformed MEFs [ $10^6/200$   $\mu$ l phosphate-buffered saline (PBS)] were injected subcutaneously into the flank of female athymic 7–8-week-old nu/nu mice, and tumours were allowed to develop until they reached 10mm<sup>3</sup>. Tumours were measured externally with a caliper, in two dimensions, on the indicated days. Tumour volumes were determined from the equation  $V = (L \times W^2) \times 0.5$ , where L is length and W is width.

#### Quantitative RT-PCR

Total RNAs were extracted from polyps and normal tissue by immersing the sampled tissue in 1 ml of TRIzol in "Lysing Matrix D" tubes by MP Bio. The tissues were lysed by placing the tubes in MP Bio's "FastPrep24" machine, for 6 cycles of 30 seconds at maximum power, interspersed with 5 minutes incubation on ice. After the lysis of the tissues, the RNAs were separated in the aqueous phase by the addition of 1-bromo-3- chloropropane, then precipitated in isopropanol, washed and resuspended in nuclease-free water. The residual genomic DNA was removed using the "DNA-free" kit from Ambion according to the manufacturer's instructions. The cDNAs were synthesized using the "High Capacity cDNA Reverse Transcription" kit from Applied Biosystem. The qPCRs were performed on a Bio-Rad CFX96 machine, using Applied Biosystem's "PowerSYBR".

#### Mice

*Ubc9<sup>fl/-</sup>* mice have been described in Nacerddine *et al.*, 2005. The *Ubc9<sup>fl/-</sup> Rosa26CreERT2* have been described in Demarque *et al.*, 2011. The *Villin-CreERT2/Apc<sup>fl/+</sup>* have been described in Andreu *et al.*, 2005. The AOM (A5486 Sigma) was injected into the peritoneum of 6-8 week old mice at a dose of 100 mg/g. The DSS (40,000 g/mol, AppliChem) was administered in the drinking water at a concentration of 1,5%, and prepared freshly for each treatment. For loss of *Apc*, 8-12weeks mice were injected with 1mg/mouse of 4-hydroxytamoxifen (H6278 Sigma) 3 times in 2 days. For the detection of the Lgr5+ stem

cells, the *Ubc9<sup>+/+</sup>* and *Ubc9<sup>+/-</sup>* mice were crossed with Lgr5-EGFP-IRES-CreERT2 (provided by the animal facility of Pasteur Institute, international name: C57BL/6J-Lgr5tm1(cre/ERT2)Cle Ube2itm). The mice were bred at the Pasteur Institute animal facility. The animal studies were conducted in accordance with French law and followed the institutional recommendations in effect. They have been validated by the ethics committee of animal experimentation of the Pasteur Institute 2016-0025.

#### Mouse histology

The sampled tissues were immersed in a 10% formalin solution at pH 7, overnight, then rinsed with water and stored in 70% ethanol at 4°C until embedding them in paraffin, then cut into 4µm sections. The sections were stained using a hematoxylin-eosin solution and analyzed by Dr. Grégory Jouvion of the group of Fabrice Chrétien, "Human Histopathology and Animal Models" at the Pasteur Institute.

#### Detection of Lgr5+ stem cells

The intestines of *Ubc9<sup>+/+</sup>/Lgr5-EGFP-IRES-CreERT2* and *Ubc9<sup>+/-</sup>/Lgr5-EGFP-IRES-CreERT2* mice were flushed with PBS and fixed overnight at 4°C in PLP-buffer (containing PFA, NaIO<sub>4</sub> (sodium periodate), L-Lysine and P-buffer). After the fixation the tissues were washed and placed in 30% sucrose overnight at 4°C. The following day the tissues were frozen using OCT (4583 Tissue-Tek) and stored at -80°C. The tissues were cut into sections and analyzed by Dr. Saskia Ellenbroek of the group of Jacco van Rheenen, "Cancer Biophysics" at the Hubrecht Institute.

#### Microarray analysis

Total RNA was collected using TRIzol extraction. RNA integrity and concentration were evaluated using 2100 Bioanalyzer and RNA 6000 Nano kit from Agilent and RNA concentration was evaluated using. Three biological replicates were analyzed for each condition. For each array, the cell intensity files (CEL) were generated with an Affymetrix GeneChip Scanner 3000. Gene-level expression values were derived from the CEL file probe-level hybridization intensities using the model-based Robust Multichip Average algorithm (RMA). Statistical analysis to compare replicates' arrays was done with the local poor error

test. The P-values were adjusted using the Bonferroni algorithm and a threshold of  $P < 0.05$  is used as the criterion for expression.

### Expression microarray pre-processing

Raw Affymetrix Mouse Gene 2.0 ST array intensity data were analyzed using open-source Bioconductor packages on R. The data were normalized all together using the robust multi-array average normalization approach implemented in the oligo package (Carvalho and Irizarry, 2010). Internal control probe sets were removed and average expression deciles over conditions were then defined. Probes whose average expression was lower than the 4th expression decile were removed for subsequent analyses. To remove sources of unwanted variation and consider batch effects, data were finally corrected with the sva package. As information as possible, we combined Affymetrix Mouse Gene 2.0 ST annotations provided by Affymetrix and Ensembl through the packages pd.mogene.2.0.st and biomaRt (Durinck et al., 2009). Principal component analysis and bi-clustering based on Pearson's correlation and Ward's aggregation criterion were used to check for consistency between biological replicates and experimental conditions at each step of the pre-processing.

### Differential expression analysis

Normalized log-scaled and filtered expression data were further considered for differential analysis with limma (Ritchie et al., 2015). Briefly, a nested general linear model considering tissue (normal or polyp), genotype (wild type, or heterozygous) and their interaction was fitted to the data. Moderated F-statistics that combines the empirical Bayes moderated t-statistics for all contrasts into an overall test of significance for each probe were used to assess the significance of the observed expression changes for each contrast. P-values were corrected for multiple testing using the FDR approach for a significance level of 0.1. A cut-off in log<sub>2</sub> fold-change at 1.5 was finally applied.

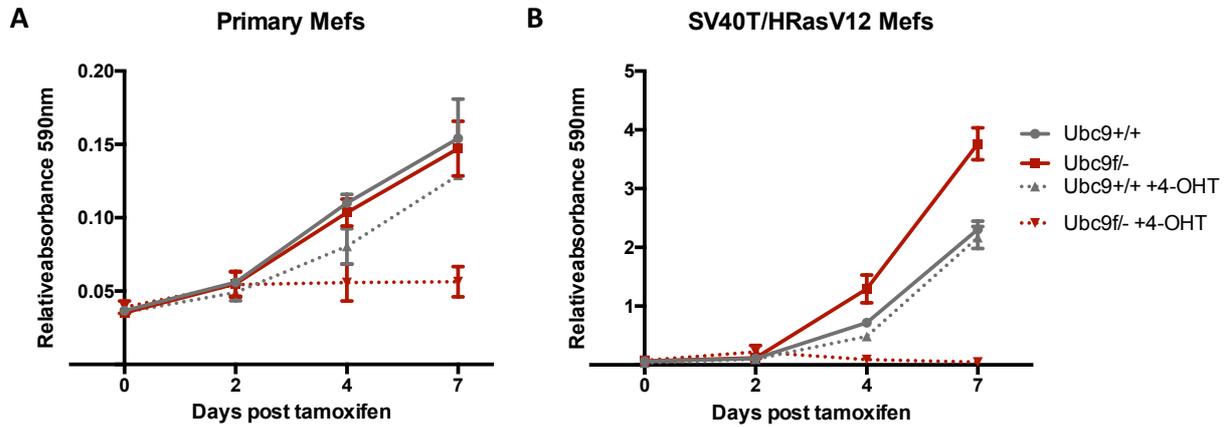
### Functional characterization

We first performed a gene set over-representation analysis for each differentially expressed gene lists considering different molecular signatures (MSigDB hallmark, MSigDB Reactome, MSigDB Biocarta, MSigDB GO BP, MSigDB Oncogenic Signatures, MSigDB

Immunologic Signatures) using a right-tail modified Fisher's exact test and the hypergeometric distribution to provide p-value. Using MSigDB curated signatures as well as a custom Stem Cell signature, we finally ran a gene set enrichment analysis using the GSEA package.

# **ANNEXES**

**Annex 1 Growth curves.** **A:** Primary *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> MEFs (pool of 5 embryos) upon 0, 2, 4 and 7 days of treatment with 4-OHT or not. **B:** *Ubc9*<sup>+/+</sup> and *Ubc9*<sup>fl/-</sup> SV40T/HRasV12 transformed MEFs, upon 0, 2, 4 and 7 days of treatment with 4-OHT or not. Mean  $\pm$  SD, n=3. Red: *Ubc9*<sup>+/+</sup>, Grey: *Ubc9*<sup>fl/-</sup>



**Annex 2 Pathways specifically enriched or depleted in Ubc9<sup>+/-</sup> polyps derived from APC loss.**

ID	NES	p.adj
PID_AP1_PATHWAY	-1.91	0.1134
PID_FAK_PATHWAY	-1.82	0.1134
NABA_MATRISOME_ASSOCIATED	1.51	0.1134
REACTOME_SIGNALING_BY_TGF_BETA_RECEPTOR_COMPLEX	-1.74	0.1134
REACTOME_TRANSCRIPTIONAL_ACTIVITY_OF_SMAD2_SMAD3_SMAD4_HETEROTRIMER	-1.74	0.1134
BIOCARTA_TNFR1_PATHWAY	-1.73	0.1134
KEGG_ARACHIDONIC_ACID_METABOLISM	1.81	0.1134
NABA_SECRETED_FACTORS	1.73	0.1134
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	1.69	0.1134
REACTOME_CELL_CYCLE	-1.55	0.1134
KEGG_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	1.83	0.1134
REACTOME_CELL_CYCLE_MITOTIC	-1.60	0.1134
REACTOME_GPCR_LIGAND_BINDING	1.72	0.1134
REACTOME_CLASS_B_2_SECRETIN_FAMILY_RECEPTORS	1.64	0.1134
REACTOME_POTASSIUM_CHANNELS	1.66	0.1134
PID_SMAD2_3NUCLEAR_PATHWAY	-1.61	0.1788
KEGG_CELL_CYCLE	-1.61	0.1788
REACTOME_MITOTIC_G1_G1_S_PHASES	-1.58	0.1788
REACTOME_ACTIVATION_OF_GENES_BY_ATF4	-1.71	0.1878
REACTOME_SMAD2_SMAD3_SMAD4_HETEROTRIMER_REGULATES_TRANSCRIPTION	-1.77	0.1878
REACTOME_MUSCLE_CONTRACTION	-1.62	0.1878
REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	1.71	0.1878
KEGG_PRIMARY_IMMUNODEFICIENCY	1.81	0.1878
KEGG_STARCH_AND_SUCROSE_METABOLISM	1.90	0.1878
REACTOME_SMOOTH_MUSCLE_CONTRACTION	-1.67	0.1878
PID_HIF1_TFPATHWAY	-1.62	0.1878
REACTOME_S_PHASE	-1.61	0.1878
PID_AR_PATHWAY	-1.64	0.1878
PID_RAC1_REG_PATHWAY	-1.66	0.1878
KEGG_TGF_BETA_SIGNALING_PATHWAY	-1.57	0.1878
KEGG_TIGHT_JUNCTION	-1.57	0.1878
REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	1.65	0.1878
REACTOME_CLASS_A1_RHODOPSIN_LIKE_RECEPTORS	1.56	0.1878
REACTOME_SYNTHESIS_OF_PA	1.82	0.1878
KEGG_ETHER_LIPID_METABOLISM	1.74	0.1878

KEGG_RETINOL_METABOLISM	1.78	0.1878
REACTOME_SIGNALING_BY_WNT	-1.59	0.1965
PID_ATF2_PATHWAY	-1.63	0.1965
KEGG_DILATED_CARDIOMYOPATHY	-1.54	0.1965
REACTOME_DNA_REPLICATION	-1.45	0.1965
REACTOME_MITOTIC_M_M_G1_PHASES	-1.44	0.1965
REACTOME_LIPOPROTEIN_METABOLISM	1.72	0.1965
KEGG_NICOTINATE_AND_NICOTINAMIDE_METABOLISM	1.71	0.1965
REACTOME_ORC1_REMOVAL_FROM_CHROMATIN	-1.54	0.1995
PID_S1P_S1P2_PATHWAY	-1.61	0.1995
REACTOME_ELONGATION_ARREST_AND_RECOVERY	-1.67	0.1995
PID_AVB3_OPN_PATHWAY	-1.63	0.1995
PID_RET_PATHWAY	-1.59	0.1995

Note: NES= Normalized enrichment score, when positive shows enrichment, when negative depletion. P.adj= adjusted p-value

**Annex 3 Pathways specifically enriched or depleted in Ubc9<sup>+/-</sup> polyps derived from AOM-DSS treatment.**

Description	NES	p.adj
REACTOME_ACYL_CHAIN_REMODELLING_OF_PC	2.03	0.0964
REACTOME_AMYLOIDS	1.94	0.0964
REACTOME_RNA_POL_I_PROMOTER_OPENING	2.01	0.0964
REACTOME_RNA_POL_I_TRANSCRIPTION	1.86	0.0964
REACTOME_PACKAGING_OF_TELOMERE_ENDS	2.00	0.0964
KEGG_PRIMARY_IMMUNODEFICIENCY	-1.88	0.0964
PID_S1P_S1P3_PATHWAY	-1.75	0.0964
KEGG_PPAR_SIGNALING_PATHWAY	-1.92	0.0964
PID_EPHA_FWDPATHWAY	-1.95	0.0964
PID_P53_DOWNSTREAM_PATHWAY	-1.73	0.0964
BIOCARTA_CTCF_PATHWAY	-2.02	0.0964
REACTOME_CD28_DEPENDENT_PI3K_AKT_SIGNALING	-1.85	0.0964
REACTOME_NITRIC_OXIDE_STIMULATES_GUANYLATE_CYCLASE	-1.76	0.0964
PID_UPA_UPAR_PATHWAY	-1.84	0.0964
KEGG_PANCREATIC_CANCER	-1.88	0.0964
REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	-2.06	0.0964
BIOCARTA_IL1R_PATHWAY	-1.79	0.0964
REACTOME_INTERFERON_ALPHA_BETA_SIGNALING	-1.83	0.0964
KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	-1.84	0.0964
KEGG_CHEMOKINE_SIGNALING_PATHWAY	-1.74	0.0964
NABA_SECRETED_FACTORS	-1.78	0.0964
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	-2.17	0.0964
REACTOME_HEMOSTASIS	-1.78	0.0964
REACTOME_SIGNALING_BY_GPCR	-1.53	0.0964
NABA_MATRISOME_ASSOCIATED	-1.62	0.0974
NABA_CORE_MATRISOME	1.47	0.0986
REACTOME_MEIOTIC_RECOMBINATION	1.76	0.1013
PID_ALK1_PATHWAY	-1.70	0.1196
REACTOME_THE_ROLE_OF_NEF_IN_HIV1_REPLICATION_AND_DISEASE_PATHOGENESIS	-1.72	0.1196
KEGG_HEMATOPOIETIC_CELL_LINEAGE	-1.81	0.1196
KEGG_INTESTINAL_IMMUNE_NETWORK_FOR_IGA_PRODUCTION	-1.76	0.1196
REACTOME_CYTOKINE_SIGNALING_IN_IMMUNE_SYSTEM	-1.57	0.1196
REACTOME_PLATELET_ACTIVATION_SIGNALING_AND_AGGREGATION	-1.73	0.1196
REACTOME_CLASS_A1_RHODOPSIN_LIKE_RECEPTORS	-1.59	0.1196
REACTOME_GPCR_LIGAND_BINDING	-1.61	0.1196
KEGG_PATHWAYS_IN_CANCER	-1.48	0.1196
REACTOME_GPCR_DOWNSTREAM_SIGNALING	-1.44	0.1196
KEGG_VIRAL_MYOCARDITIS	-1.75	0.127

KEGG_BLADDER_CANCER	-1.75	0.1271
REACTOME_ENDOSOMAL_SORTING_COMPLEX_REQUIRED_FOR_TRANSPORT_ESCRT	-1.66	0.1271
REACTOME_LIPOPROTEIN_METABOLISM	-1.65	0.1271
REACTOME_COSTIMULATION_BY_THE_CD28_FAMILY	-1.80	0.1271
KEGG_MELANOMA	-1.74	0.1271
REACTOME_SIGNALING_BY_ILS	-1.60	0.1271
REACTOME_INTERFERON_SIGNALING	-1.64	0.1271
PID_S1P_S1P1_PATHWAY	-1.71	0.1271
KEGG_RIBOSOME	-1.72	0.1271
REACTOME_TELOMERE_MAINTENANCE	1.62	0.1432
REACTOME_ABC_FAMILY_PROTEINS_MEDIATED_TRANSPORT	-1.67	0.1460
PID_ARF6_PATHWAY	-1.62	0.1460
KEGG_LEISHMANIA_INFECTION	-1.66	0.1460
KEGG_TGF_BETA_SIGNALING_PATHWAY	-1.64	0.1460
REACTOME_PEPTIDE_CHAIN_ELONGATION	-1.71	0.1460
KEGG_PEROXISOME	-1.65	0.1460
KEGG_CELL_ADHESION_MOLECULES_CAMS	-1.62	0.1460
REACTOME_FATTY_ACID_TRIACYLGLYCEROL_AND_KETONE_BODY_METABOLISM	-1.51	0.1505
REACTOME_DEPOSITION_OF_NEW_CENPA_CONTAINING_NUCLEOSOMES_AT_THE_CENTROMERE	1.60	0.1706
REACTOME_GASTRIN_CREB_SIGNALLING_PATHWAY_VIA_PKC_AND_MAPK	-1.48	0.1709
REACTOME_INFLUENZA_VIRAL_RNA_TRANSCRIPTION_AND_REPLICATION	-1.59	0.1709
REACTOME_MEIOTIC_SYNAPSIS	1.63	0.1749
REACTOME_RNA_POL_I_RNA_POL_III_AND_MITOCHONDRIAL_TRANSCRIPTION	1.54	0.1749
REACTOME_PLATELET_HOMEOSTASIS	-1.64	0.1869
REACTOME_CD28_CO_STIMULATION	-1.60	0.1869
REACTOME_3_UTR_MEDIATED_TRANSLATIONAL_REGULATION	-1.55	0.1882
KEGG_MATURITY_ONSET_DIABETES_OF_THE_YOUNG	1.68	0.1891
REACTOME_LIPID_DIGESTION_MOBILIZATION_AND_TRANSPORT	-1.58	0.1935
KEGG_GLIOMA	-1.53	0.1935
PID_HIF1_TFPATHWAY	-1.53	0.1935
REACTOME_CTLA4_INHIBITORY_SIGNALING	-1.62	0.1935
REACTOME_EFFECTS_OF_PIP2_HYDROLYSIS	-1.64	0.1935
REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	-1.55	0.1935
REACTOME_METABOLISM_OF_LIPIDS_AND_LIPOPROTEINS	-1.28	0.1952

Note: NES= Normalized enrichment score, when positive shows enrichment, when negative depletion. P.adj= adjusted p-value

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### **Exploring the role of SUMOylation in cellular transformation and colorectal cancer**

The post-translational modification by the Small Ubiquitin-like MOdifier (SUMO) is an essential regulatory mechanism of protein function affecting the interactions of its substrates with their protein and nucleic acid partners, their localization, stability or enzymatic activity. SUMOylation is a reversible process involving specific enzymes. Using genetically modified models deficient for the unique E2 enzyme, UBC9, we characterized the consequences of hypoSUMOylation in cellular transformation and colorectal cancer.

The results obtained *in vitro* demonstrate that a total loss of SUMOylation does not have significant effects in the survival of primary cells but strongly affects the survival of transformed cells. Surprisingly however, the reduction in half of the quantity of UBC9 increased the proliferation of transformed cells but had no effect in the proliferation of primary cells.

In animal models, contradictory results were obtained. More specifically, the reduction in half of the quantity of UBC9 increased the number of intestinal polyps in a murine model of genetically induced colorectal carcinogenesis (APC). On the contrary, in a chemical model consisting of mutagenic and an inflammatory agent (AOM-DSS), the reduction in half of UBC9 decreases the number of polyps in the colon of the mice.

On the whole, these results allow us to increase our knowledge of SUMOylation and to envisage a possible use of SUMOylation inhibitors for cancer therapy, however the level of SUMOylation would have to be strictly and finely controlled depending on the type of cancer.

### **Étude du rôle de la SUMOylation dans la transformation cellulaire et le cancer colorectal**

La modification post-traductionnelle par SUMO régule des mécanismes essentiels des fonctions protéiques comme leurs interactions avec des protéines ou des acides nucléiques, leurs localisations subcellulaires, leurs stabilités ou encore leurs activités enzymatiques. La SUMOylation est un processus réversible qui fait intervenir des enzymes spécifiques. À l'aide de modèles murins génétiquement modifiés déficient pour l'enzyme unique E2, UBC9, nous avons pu caractériser les conséquences de l'hypoSUMOylation sur la transformation des cellules et l'oncogenèse colorectal.

Les résultats obtenus *in vitro* montrent qu'une perte totale de la SUMOylation n'a pas d'effet significatif sur la survie des cellules primaires mais affecte très fortement celle des cellules transformées. De manière surprenante, la réduction de moitié de la quantité d'UBC9 augmente la prolifération des cellules transformées mais n'a aucun effet sur la prolifération des cellules primaires.

Dans les modèles animaux des résultats contradictoires ont été obtenus. En effet, la réduction de moitié de la quantité d'UBC9 augmente le nombre de polypes intestinaux dans un modèle murin de cancer colorectale induit génétiquement (APC). Par contre, dans un modèle chimio-induite en présence d'un agent inflammatoire (AOM-DSS), la réduction de la quantité d'UBC9 réduit le nombre de polypes dans les colons des souris.

Ces résultats permettent d'affiner nos connaissances sur le rôle de la SUMOylation et d'envisager que des inhibiteurs de la SUMOylation pourraient être utilisées en thérapeutique. Néanmoins, le niveau de SUMOylation devra alors être très strictement et finement contrôlé en fonction du type de cancers.