Final report
SAW-2014-FLI-2 311 DNA damage responses in aging

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Table of contents

1. Executive summary

Increasing evidence indicates that the accumulation of nuclear DNA damage represents one of the molecular causes of aging. Accordingly, there is an age-dependent accumulation of DNA damage in numerous human tissues. Genetic diseases that lead to premature aging are often caused by mutations in genes involved in DNA damage repair, and animal models provided a proof of concept that DNA damage accumulation leads to premature aging. Cells respond to DNA damage by activating checkpoints that prevent the contribution of damaged cells to tissue homeostasis by induction of cell death (apoptosis), cell cycle arrest (senescence) or self-digestion of damaged molecular and cellular components (autophagy). There is evidence that the induction of a DNA damage response (DDR) prevents cancer formation by eliminating genetically instable cells. However, when a growing number of cells in aging tissues accumulates DNA damage, the induction of the same responses can also lead to loss of tissue maintenance and tissue atrophy. In agreement with this conceptual view, we provided a proof of principle that the deletion of genetic components of the DDR in aged tissues with a high load of DNA damage can prolong stem cell function, tissue maintenance, and lifespan. The main aims of this proposal were to delineate the functional influence of the DDR on cellular and tissue aging at different levels including the role of (i) End resection of damaged DNA in the initiation of DDRs, (ii) upstream mediators of DDR signaling components such as the ATR and ATM kinases, and (iii) downstream consequences of chronic DDR activation, e.g. the senescence associated secretory phenotype (SASP). It was planned to study the role of these processes in different organs and subcellular compartments and processes within a network of collaborating Leibniz Institutes (FMP, IUF, LIN). Collaboration within the network was planned to identify chemical compounds that interfered with molecular pathways promoting tissues aging in response to DNA damage. Results from the Rudolph group determined *H2ax* as an inhibitor of end resection and chromosomal fusion formation in response to telomere shortening. *53bp1* was identified as mediator of chromosomal fusions and the deletion of *53bp1* reduced DNA damage and tissue atrophy and elongated the lifespan of telomere dysfunctional mice. Instead, the deletion of *Polq* - a mediator of alternative end joining - was not sufficient to prevent chromosomal fusions and premature aging of telomere dysfunctional mice. Together, these result point to novel therapeutic target to prevent chromosomal fusion and tissue atrophy in the context of telomere shortening. The Kaether group optimized a secretion inhibitor by structure-activity relation studies and tested for its suitability to inhibit SASP. A proof-of-concept was done, but the compound itself induced senescence. Important experience was gained in preparation for high-throughput screens for novel SASP inhibiting compounds and cellular mediators of SASP that will enable to conduct these screens in future studies. The Wang group carried out genetic, cellular and molecular analyses of DNA damage response, governed by DDR molecules NBS1 and ATR using cellular and animal models. They also applied proteomic and metabolic studies to dissect the molecular pathways affected by the malfunctional DDR in neural cells including postmitotic neurons. Our studies show that the classical DDR molecules play a physiological function in postmitotic cells and thereby in tissue homeostasis.

Altogether, the current project has improved our understanding of the role of DNA damage response in driving cellular dysfunction, tissue degeneration, and disease development during aging. Breakthrough results included the identification of new genetic components that mediate DDRs and tissue atrophy in response to telomere shortening, new compounds that inhibit SASP - a disease promoting condition in aging tissues, and new insights into the role of upstream DDR components in aggravating tissue decline and neurodegeneration. These findings provide a knowledge basis for the development of therapies aiming to facilitate healthy aging by ameliorating DNA damage responses.

2. Rationale and Aims of the Proposed Project

DNA damage accumulates in aging cells and tissues. While protecting against cancer formation at young age, the activation of DNA damage responses (DDRs) can lead to impairments in organ homeostasis in aging tissues harboring a growing number of cells that carry DNA damage. Based on proof of concept experiments from our consortium we pursue the central **hypothesis that targeted impairments of the DDR can prolong functionality of cells and organs during aging. In the current project,** we tested this hypothesis using mouse models of DNA damage induced aging. We collaborated in a network of Leibniz institutes (FMP, IUF, LIN) aiming to delineate consequences of DDRs (i) on stem cell aging and the maintenance of proliferative tissues, (ii) on the maintenance of differentiated, non-dividing neurons, and (iii) on subcellular structures (mitochondria) and cellular processes (apoptosis, senescence, autophagy) involved in aging. Together, these studies should provide a rational basis to begin to develop molecular therapies aiming to prolong "healthy aging" by impairing DDRs that promote the atrophy and dysfunction of aged tissue.

3. Results Work Program

3.1. Targeting DNA end resection to slow tissue aging in response to DNA damage and telomere dysfunction *(Rudolph)*

Telomere shortening limits the proliferative capacity of cells and organ maintenance during aging (Rudolph et al. 1999). It has been shown that telomere shortening leads to a loss of telomere capping of chromosomal ends resulting in the activation of DNA damage responses (DDRs), which result in chromosomal fusion and the activation of *p53* mediated senescence (permanent cell cycle arrest) and apoptosis (Sperka et al. 2011). In previous work, we showed that deletion of Exonuclease-1 (*Exo1*) impairs the resection of DNA and the induction of fusions and DDRs in aging telomerase knockout mice with dysfunctional telomeres (Schaetzlein et al. 2007). Of note Exo1 deletion improved organ maintenance and elongated the lifespan of telomere dysfunctional mice (Schaetzlein et al. 2007). Together, these results provided a proof of concept that the inhibition of end-resection prevents the induction of chromosomal fusions and DDRs and thus represents a therapeutic target to improve organ maintenance in aged tissues with shortened telomeres.

3.1.1. Development of the research program and presentation of the results in the context of the relevant literature

Within the SAW-project on "DDRs in aging" we worked on 3 main aims:

3.1.1.1. Delineate DNA end-resection dependent pathways that promote tissue aging in response to DNA damage.

In our work within the SAW-project, we analysed the functional consequences of *H2ax* deletion, *53bp1* deletion, and *Polq* deletion on the development of tissue atrophy and lifespan shortening in late generation telomerase knockout mice (iG4 *mTerc*$^{-/-}$ mice or G3 *mTerc*$^{-/-}$ mice) with dysfunctional telomeres. *H2ax* encodes for a histone variant, which is phosphorylated in response to DNA damage.

Phosphorylated of H2AX (aka γH2AX) is one of the earliest steps in DDR pathways and leads to recruitment of MDC1, ATM activation and inhibition of end-resection. In our studies, the knockout of *H2ax* in the intestinal epithelium aggravated the atrophy of the intestinal epithelium and shortened the lifespan of iG4 *mTerc-/-* mice with dysfunctional telomeres (Omrani, O. PhD thesis, submitted). These results are in agreement with our previous study indicating that DNA end-resection contributes to the induction of DDRs, chromosomal fusions and tissue atrophy in aging, telomere dysfunctional mice Schaetzlein et al. 2007).

Similar to H2AX, 53BP1 is involved in blocking end-resection in response to DNA damage. It has been shown that 53BP1 promotes non-homologous end-joining (NHEJ) mediated DNA repair by blocking end resection and by tethering of chromosome ends (Bothmer et al. 2010). In response to DNA damage, 53BP1 protein is phosphorylated by ATM and locates to sites

of DNA breakage within 30 minutes after damage induction. Our study within the SAW-project revealed that *53bp1* deletion impairs induction of DDRs and chromosomal fusion formation in the intestinal epithelium of telomere dysfunctional mice, which results in improved organ maintenance and an elongated lifespan (Fig. 3.1.1. A-C). Taken together, the results suggest that loss of inhibition of DNA end resection *per se* does not lead to an increase in DDRs, fusion formation, or tissue atrophy in response to telomere dysfunction. Instead, aggravation of telomere dysfunction induced phenotypes appears to be rescued by impairments in NHEJ in response to *53bp1* deletion (Sperka et al. and Rudolph, in preparation of submission).
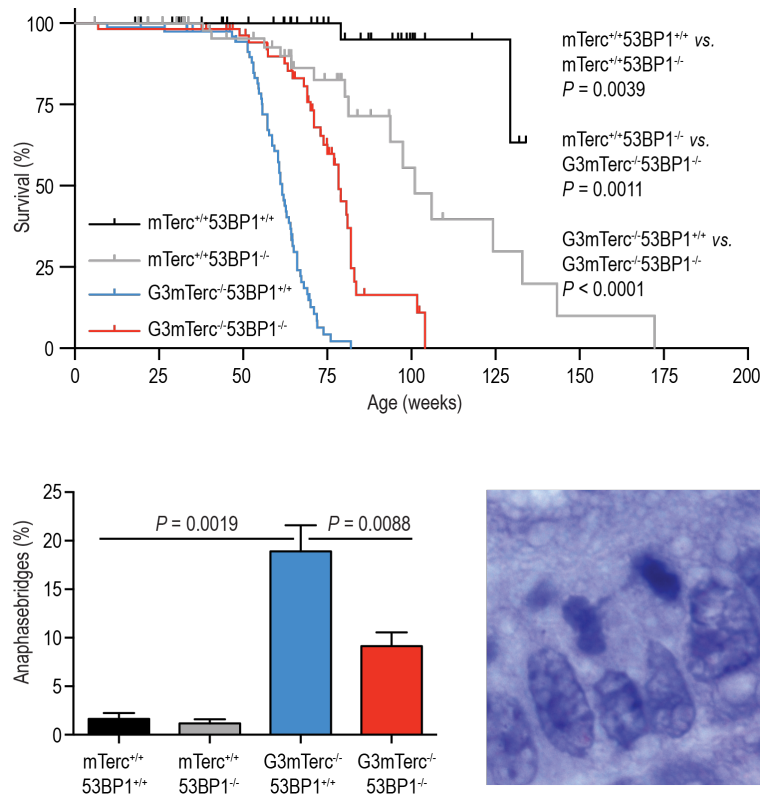

Figure 3.1.1. (**A**) Kaplan-Meier survival curve of mice of the indicated genotypes (n= 47 WT, n=80 G3 Terc-KO, n= 60 53BP1 KO, n= 58 G3 Terc, 53BP1 double KO). The lifespan of G3 mTerc-/- mice with dysfunctional telomeres is shortened compared to mTerc+/+ mice. 53bp1 knockout rescues survival of G3 mTerc-/- mice. (**B**) The number of anaphase bridges (a morphological hallmark of chromosomal fusion, as shown in the representative image) was quantified as a percentage of all analyzed anaphases in the intestinal epithelium of 10-14 month old mice of the indicated genotypes. Note that there was an increase in anaphase bridges in G3 mTerc-/- mice with dysfunctional telomeres compared to mTerc+/+ mice but the 53bp1 deletion led to a significant reduction of the number of anaphase bridges.

The *Polq* gene encodes the DNA polymerase theta (Polθ), which has been implicated in alternative end-joining (A-EJ) of dysfunctional telomeres (Mateos-Gomez et al. 2015). There has been a debate whether classical NHEJ (c-NHEJ) or A-EJ may be involved in fusion formation in response to telomere shortening, since it has been shown that deletion of c-NHEJ components, such as DNA-PKcs, cannot rescue fusion formation and tissue atrophy in telomerase deficient mice with short, dysfunctional telomeres (Maser et al. 2006). To analyse the possible contribution of A-EJ, *Polq* knockout mice were crossed with *mTerc*-deficient mice. The study revealed that *Polq* deletion does not rescue induction of DDRs, the formation of chromosomal fusion or the progressive tissue atrophy and early death of telomere dysfunctional mice (Fig. 3.1.2. A-C, Huber, in preparation for thesis and Huber and Rudolph in preparation for paper submission).
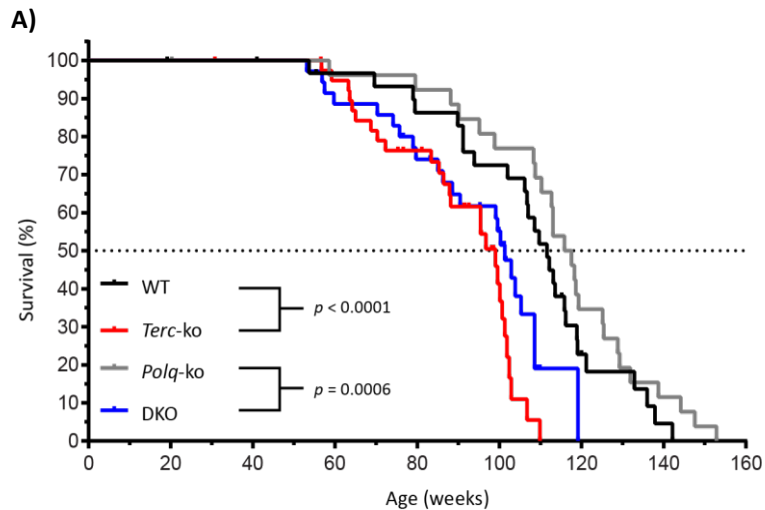
A)

Figure 3.1.2.
(**A**) Kaplan-Meier survival curve of mice of the indicated genotypes (n= 32 WT, n=40 Terc-KO, n= 27 Polq KO, n= 35 double knockout (DKO)). The lifespan of mTerc-/- mice with dysfunctional telomeres is shortened compared to wildtype (WT) mice. Polq deletion has no significant impact on the lifespan of either of the cohorts. (**B**) The number of anaphase bridges (a morphological hallmark of chromosomal fusion, as shown in the representative image) was quantified as a percentage of all analyzed anaphases in the intestinal epithelium of 19-33 month old mice of the indicated genotypes (n= 7-9 mice per group). Note, that there was an increase in anaphase bridges in mTerc-/- mice with dysfunctional telomeres compared to WT mice but the Polq gene status had no significant effect on the number of anaphase bridges. (**C**) DDRs lead to p53-dependent induction of the cell cycle inhibitor p21. The histogram shows the percentage of intestinal basal crypts that stain positive for p21 expression in 21-31 month old mice of the indicated genotypes (n= 5 mice per group). Note that the intestinal epithelium of mTerc-/- mice shows an increase in p21 expression, which is not rescued by Polq deletion.

B)

C)
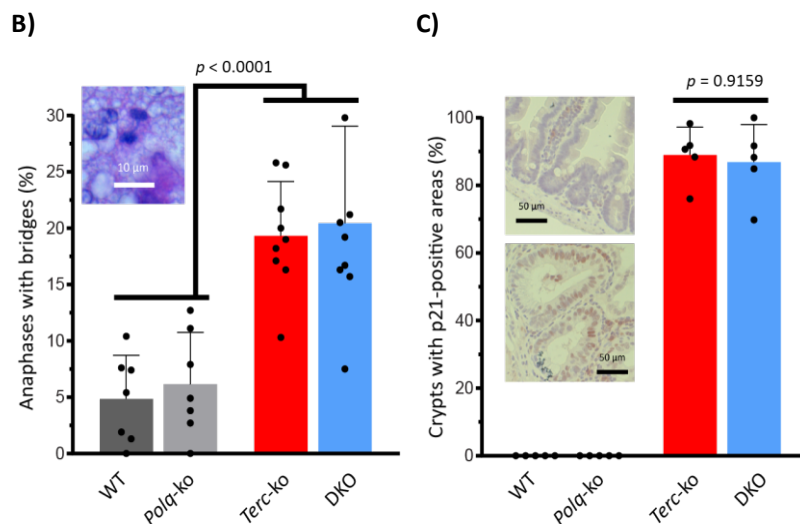
Together, the results within the SAW-project "DDRs in aging" have improved our understanding on the role of end resection and fusion formation in promoting DDRs, tissue atrophy and lifespan shortening in response to telomere shortening. The results indicate that increases in end resection aggravate aging phenotypes of telomere dysfunctional mice but the aggravation of aging phenotypes appears to depend on the formation of chromosomal fusions, which are inhibited by *53bp1* deletion but unaffected by *Polq* deletion.

3.1.1.2. Development of chemical inhibitors of end resection

and

3.1.1.3. Role of DNA end resection dependent DDR in inhibiting mitochondrial function and autophagy in aging tissues

Within the SAW-project, we decided to focus our efforts on the first aim (3.1.1.1.), because we wanted to narrow down the most promising targets in DNA end resection and DDR pathways to be explored for the development of chemical inhibitors (aim 3.1.1.2) or for studying its effects on mitochondrial function and autophagy in aging tissues (aim 3.1.1.3.). We had learned from our work on p21-inhibition that the amount of experiments is exponentially increasing once it comes down to the validation of chemical compounds. Our previous project on chemical inhibitors on p21 had failed at the stage of candidate compound validation (Hoffman and Rudolph, unpublished). Therefore, we decided to fully focus on the identification of candidate pathway components of DNA end resection and end joining before starting

a new compound screen. Following this line of argumentation, we focussed our work for aim 3.1.1.2. on redesigning cell culture-based screens to identify inhibitors of p21. Instead of screening for inhibitors of p53-dependent, transcriptional induction of p21 in a colon cancer cell line, we now generated N-terminal and C-terminal p21-GFP fusion protein knockins (into the p21 locus) of a primary, telomerase-immortalized, human fibroblast cell line (BJ). These cells will now be employed for a new screen on p21 inhibitors, which should reveal compounds that either affect protein stability or transcriptional induction of p21 in a primary, non-cancerous cell line. Based on our results of the SAW project, we will start to design new compound screens to inhibit *53bp1* dependent fusion formation or *Exo1* dependent end resection in response to telomere shortening. We will also start in our future work to analyse the consequences of inhibiting these pathway components on metabolism and autophagy of telomere dysfunctional cells.

3.1.2. Statement whether the current results are of interest for patenting or useful for starting of an industrial cooperation

The current results have deepened our understanding, which signaling pathways should be targeted to rescue induction of chromosomal fusions, DDRs, and tissue atrophy in telomere dysfunction induced aging. This knowledge as such is not patentable but will guide our conductance of compound screens to specifically interfere with these signaling pathways. We anticipate that candidate compounds that interfere with *Exo1* dependent end resection of *53bp1* mediated formation of chromosomal fusions, will be promising target for patenting compounds aiming to improve tissue maintenance in the context of aging and telomere dysfunction induced organ failure.

3.1.3. Statement on the contribution of external cooperation partners of the described projects

The project was fully conducted in our own laboratory. We plan to involve cooperation partners from the FMP in Berlin to conduct compound screens on p21-inhibitors on our newly generated p21-GFP fusion protein reporter cell lines as well as for chemical modifications of candidate compounds from theses screens.

3.1.4. PhD theses and manuscripts in preparation

Omrani, O. (PhD thesis submitted): H2ax deletion aggravates chromosomal fusions, DNA damage responses an premature aging of telomere dysfunctional mice.

Huber, N. (PhD thesis and paper in preparation): PolΘ deletion does not rescue chromosomal fusion formation, DNA damage response, or premature aging of telomere dysfunctional mice.

Sperka, T. (paper in preparation): 53bp1 deletion rescues chromosomal fusions, DNA damage responses and ameliorates premature aging of telomere dysfunctional mice.

3.1.5.References (own references in italics)

Bothmer A, Robbiani DF, Feldhahn N, Gazumyan A, Nussenzweig A, Nussenzweig MC. 53BP1 regulates DNA resection and the choice between classical and alternative end joining during class switch recombination. J Exp Med. 2010; 207:855-65.

Maser RS, Wong KK, Sahin E, Xia H, Naylor M, Hedberg HM, Artandi SE, DePinho RA. DNA-dependent protein kinase catalytic subunit is not required for dysfunctional telomere fusion and checkpoint response in the telomerase-deficient mouse. Mol Cell Biol. 2007:2253-65.

Mateos-Gomez PA, Gong F, Nair N, Miller KM, Lazzerini-Denchi E, Sfeir A. Mammalian polymerase θ promotes alternative NHEJ and suppresses recombination. Nature. 2015; 518:254-7.

Rudolph KL, Chang S, Lee H-W, Blasco M, Gottlieb GJ, Greider C, and DePinho RA. Longevity, stress response and cancer in aging telomerase deficient mice. Cell 1999; 96:701-712.

Schaetzlein S, Kodandaramireddy NR, Ju Z, Lechel A, Stepzynska A,Lilli DR, Clark AB, Rudolph C, Kuhnel F, Wei K Schlegelberger B, Schirmacher P, Kunkel TA, Greenberg RA, Edelmann W, Rudolph KL. Exo1 deletion impairs DNA damage signalling and prolongs lifespan of telomere dysfunctional mice. Cell 2007, 130:863-77

Sperka T, Song Z, Morita Y, Nalapareddy K, Guachalla LM, Lechel A, Begus-Nahrmann Y, Burkhalter MD, Mach M, Schlaudraff F, Liss B, Ju Z, Speicher MR, Rudolph KL. Puma and p21 represent cooperating checkpoints limiting self-renewal and chromosomal instability of somatic stem cells in response to telomere dysfunction. Nat Cell Biol. 2011, 14:73-9.

3.2. Targeting the secretory pathway to improve tissue maintenance in old age (Kaether)

The accumulation of DNA damage and telomere dysfunction leads to the induction of senescence. Senescent cells lose their proliferative capacity and show an enhanced secretion of pro-inflammatory cytokines, which is also known as the "senescence associated secretory phenotype (SASP)". It has been shown that SASP affects neighboring, non-senescent cells and this may impact on tissue aging and cancer formation. We hypothesized in the proposal that inhibition of SASP could delay tissue dysfunction and extend healthspan.

Little is known about the SASP-underlying changes in structure and composition of the secretory pathway. Senescence is associated with morphological changes in the secretory pathway like dispersal of the Golgi, increase in lysosomal volume, increase in lysosomal lipofuscin aggregation and increased expression of the lysosomal enzyme β-galactosidase.

We proposed 3 aims: i) to analyze our secretion inhibitor FLI-06 for its potential to inhibit SASP, ii) to develop a screen for novel inhibitors and iii) to screen for novel players involved in SASP that can be used as novel targets.

3.2.1 Analyze the potential of FLI-06 and its derivatives in inhibiting the SASP.

We proposed to extend our studies on FLI-06. The initial study was published (Krämer et al., 2013) and an extensive structure-activity relationship (SAR) was performed (manuscript in preparation). More than 140 derivatives were synthesized and tested and functional groups of the molecule were characterized. It was not possible to get a compound that is active in the nanomolar range, but we could isolate some selected active derivatives with EC50 values around 1-1.5 μM. We then established assays to induce senescence in MRC5 cells with doxorubicin, and as proposed tested to what extend FLI-06 inhibits SASP. As read-out we used the SASP component IL-6 (Fig. 3.2.1). As expected, senescent cells indeed secreted more IL-6 than normal cells (Fig. 1C). FLI-06 reduced the secretion of IL-6 in senescent cells (Fig. 1D), but to a much lesser extend than BFA, a well-established secretion inhibitor. We also noted that in normal cells treatment with FLI-06 increased IL-6, which is not explainable at the moment (FLI-06 inhibits general secretion in all cell lines tested). We also tested other SASP components and found that FLI-06 reduced their secretion (Fig. 3.2.2).
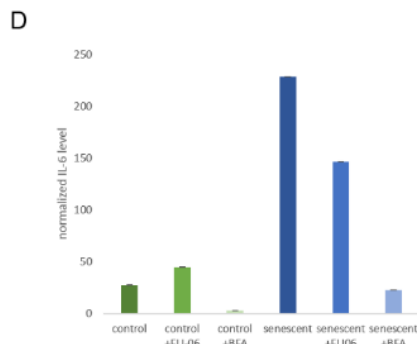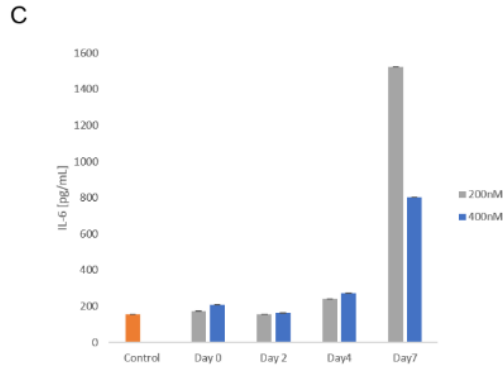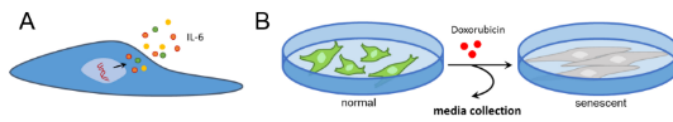
Fig. 3.2.1: FLI-06 reduces IL-6 secretion. (A) IL-6 is a common SASP factor. (B) Graphical illustration of experimental set-up: Media samples were collected during senescence induction and analysed by ELISA of IL-6. (C) Quantification of IL-6 secretion in control, 200 and 600nM Doxorubicin-treated MRC5 cells at day 0, 2, 4 and 7. Error bars indicate standard deviation. (D) Quantification of IL-6 secretion by ELISA. Control cells and senescent cells (treated with 400nM Doxorubicin for 9 days) were incubated with or without 10µM FLI-06 or 1µg/mL BFA for 20h. Error bars are indicating the standard deviation. Detected IL-6 levels (D) (measured by ELISA; pg/mL) were normalized with protein concentrations of cell lysates, as cell numbers differed between the distinct conditions
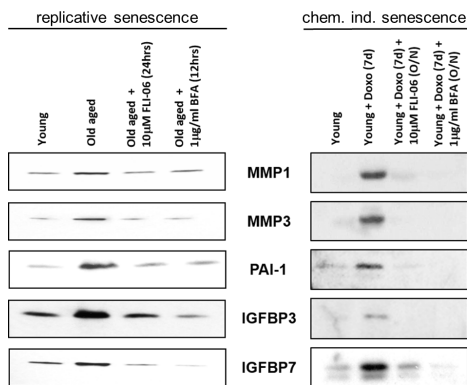

Fig. 3.2.2: FLI-06 inhibits secretion of different SASP factors in MRC-5 cells. Western blots of medium samples from MRC-5 cells collected over 4 h in serum-free medium are shown. Depicted are representative blots for MMP1, MMP3, PAI-1, IGFBP3 and IGFBP7. Samples from replicative senescent cells (old aged, PD 60-70) with and without FLI-06 or BFA treatment, as well as doxorubicin treated cells with and without additional FLI-06 or BFA treatment were examined, while samples of young cells (PD ~32) were used as negative control.

We then moved on and tried to establish a read-out system for inhibition of SASP using the secreted alkaline phosphatase (SEAP). A lentiviral construct expressing SEAP was generated and senescent and non-senescent cells were infected. Fig. 3.2.3A shows that SEAP activity was measurable in infected, but not in control cells, both in senescent and non-senescent cells. Unfortunately, this activity could not or only slightly be blocked by FLI-06 (Fig. 3.2.3B), although we successfully used SEAP secretion in combination with FLI-06 inhibition in other cells (Yonemura et al., 2016). Moreover, we discovered that FLI-06 itself is an effective inducer of senescence (Fig. 3.2.4).
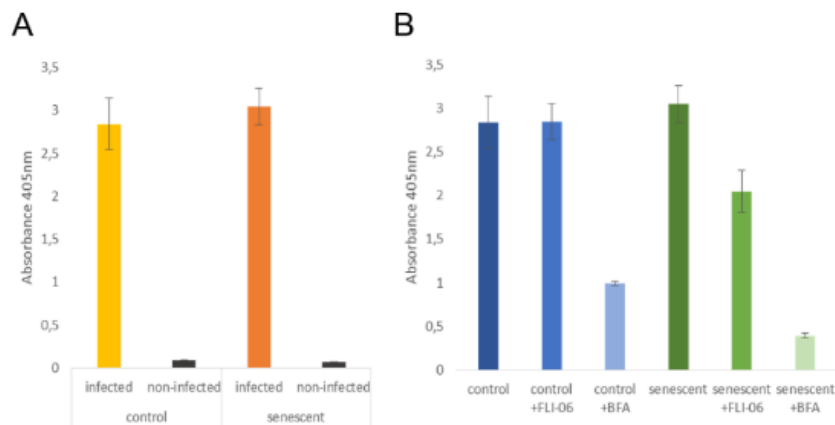
Fig. 3.2.3: FLI-06 inhibits the secretion of SEAP. (A) Quantification of SEAP activity by measuring absorbance at 405nm revealed a clear difference between virally infected and non-infected cells, in both senescent and non-senescent cells. Control, normal MRC5 cells; senescent, MRC5 cells treated for 7 days with 400 nM doxirubicin. (B) Quantification of SEAP activity by measuring absorbance at 405nm of normal and senescent cells treated for 18 h with 10 μM FLI-06 or 1 mg/ml BFA.
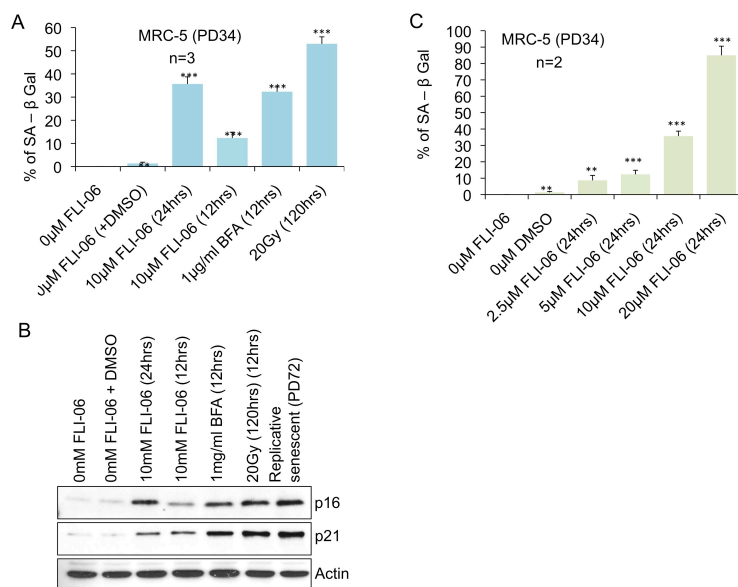

Fig. 3.2.4: FLI-06 induces senescence. A) MRC-5 cells were treated as indicated, subjected to SA-ß-Gal staining and analyzed by bright-field microscopy. Displayed is the percentage of ß-Gal-positive cells in %. B) MRC-5 cells treated as indicated were lysed and subjected to Western Blotting with indicated antibodies. P16 and p21 are markers for senescence. The actin control refers to the p16 blot. C) MRC-5 cells were treated with increasing conditions of FLI-06 as indicated, subjected to SA-ß-Gal staining and analyzed by bright-field microscopy. Displayed is the percentage of ß-Gal-positive cells in %.

Taken together, the principal proof of concept was achieved. FLI-06 can inhibit SASP and a SAR study was performed, yielding important insights into the chemistry of the compound. However, no derivative in the nanomolar range was identified. Moreover, during the experiments it turned out that FLI-06 itself induced senescence. Therefore the planned experiments in collaboration with the LIN and the FMP were not conducted. Instead, we decided to further look into the details of the rapid senescence induction by FLI-06, to understand the connection between blocking ER-export and senescence induction. To this end we performed RNAseq experiments of several primary cells with and without FLI-06 and BFA treatment. After bioinformatics analysis several individual senescent-associated genes were indeed found to be up-regulated, but we could not identify a clear link between ER-stress, ERAD, or other ER-associates processes that would give hints to a possible connection between the ER-exit inhibition and senescence induction. The experiments were conducted in connection with another study that was published (Marthandan et al., 2016a, Marthandan et al., 2016b)

3.2.2. Conduct compound screen for novel SASP inhibitors

Aim of this part was to screen novel SASP inhibitors with senescent human fibroblasts that stably express SEAP. A lentivirus expressing SEAP was constructed and tested (see Fig. 3.2.3). Different conditions were tested, to first infect young fibroblasts, select for puromycin resistance and then induce senescence, or first establish senescence and then infect cells with virus. Both approaches worked, however because FLI-06 as our positive control did not sufficiently inhibit SEAP activity in cell culture supernatants we refrained from further optimizing this assay for high throughput compound screening. More work has to be done in order to establish suitable assay conditions for high-throughput-screening.

3.2.3. Functional and genetic analysis of the SASP to identify novel targets to inhibit SASP

The aim of this part was to set up a genome-wide siRNA screen to identify novel targets involved in senescence, SASP and autophagy. We prepared lentiviral constructs for a lysosomal marker, lamp1-RFP and tested various conditions, to find out whether it is better to first infect cells and then induce senescence or the other way around, similar to the SEAP assay mentioned above. Fig. 3.2.5 shows the proof of concept, demonstrating that in senescent and non-senescent BJ cells the lysosomal system is nicely labeled after infection with the lamp1-RFP virus. Moreover, there is a clear difference in fluorescent intensity. As expected, the senescent cells display a much higher total lysosomal fluorescence, in accord with their up-regulated lysosomes. Because of experimental problems in cloning of viral constructs and handling primary cells there was not enough time to co-infect and select cells with other markers of the secretory pathway. Because the original proposed aim is still important and because the project yielded a lot of important information about viral construction, induction and handling of senescent cells, this project will be continued.
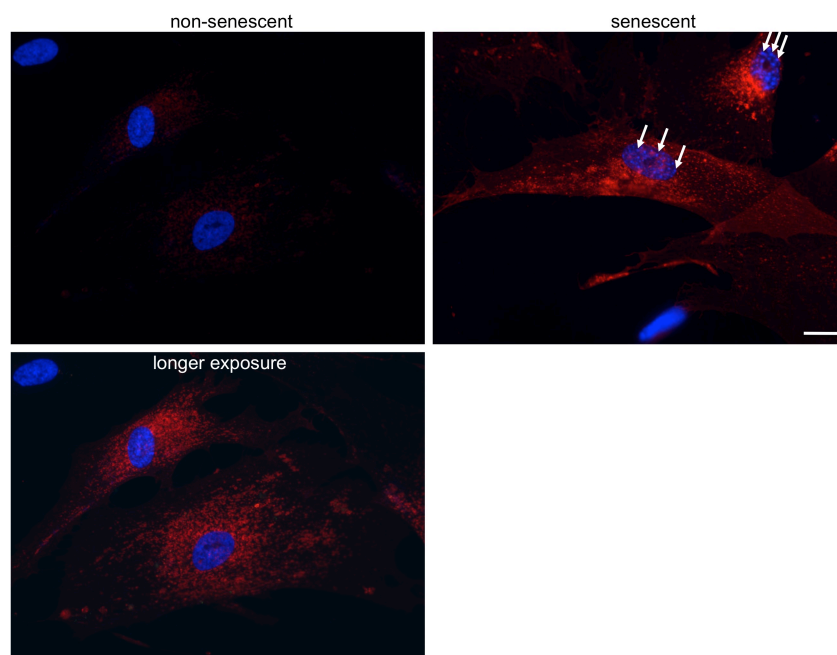

Fig. 3.2.5: Increased lysosomal content can be visualized in senescent cells. BJ cells were untreated or subjected to doxorubicin-mediated senescence induction, infected with lentiviral constructs coding for lamp1-RFP, fixed and analyzed by fluorescent microscopy. Top: same exposure time and image processing of non-senescent and senescent cells, demonstrating that senescent cells have a higher lysosomal content. Lower panel: higher exposure of non-senescent cells to demonstrate lysosomal labeling. Scale bar 10 μm.

3.2.4. Conclusion and deliverables

Taken together, the SAR study of FLI-06 resulted in very promising information about the molecule, but the experiments to test its suitability for inhibiting SASP turned out to be not satisfying. Moreover, FLI-06 itself induced senescence, an interesting finding that we begun to analyze, but which will be followed-up in future studies. Chemical and siRNA screens using senescent cells and markers of the secretory pathway turned out to be more difficult than expected. However, important experience was gained during the funding period enabling the project to be continued in future studies.

3.2.5. Manuscripts in preparation

Nohl, R. et al. Potent inhibition of ER-export: A SAR study of the dihydropyridine FLI-06

Master theses

Gisela Gassner: The effects of the secretion inhibitor FLI-06 on the senescence-associated secretory phenotype (SASP)

Anne Heiner: Investigations into the senescence-associated secretory phenotype

Talitha Feuerhake: Struktur-Aktivitäts-Beziehungen des Dihydropyridins FLI-06 – ein Inhibitor des Notch-Signalweges

3.2.6.References

Krämer A, Mentrup T, Kleizen B, Rivera-Milla E, Reichenbach D, Enzensperger C, Nohl R, Tauscher E, Gorls H, Ploubidou A, Englert C, Werz O, Arndt HD, Kaether C (2013) Small molecules intercept Notch signaling and the early secretory pathway. Nat Chem Biol 9: 731-8

Marthandan S, Baumgart M, Priebe S, Groth M, Schaer J, Kaether C, Guthke R, Cellerino A, Platzer M, Diekmann S, Hemmerich P (2016a) Conserved Senescence Associated Genes and Pathways in Primary Human Fibroblasts Detected by RNA-Seq. PloS one 11: e0154531

Marthandan S, Menzel U, Priebe S, Groth M, Guthke R, Platzer M, Hemmerich P, Kaether C, Diekmann S (2016b) Conserved genes and pathways in primary human fibroblast strains undergoing replicative and radiation induced senescence. Biol Res 49: 34

Yonemura Y, Li X, Muller K, Kramer A, Atigbire P, Mentrup T, Feuerhake T, Kroll T, Shomron O, Nohl R, Arndt HD, Hoischen C, Hemmerich P, Hirschberg K, Kaether C (2016) Inhibition of cargo export at ER exit sites and the trans-Golgi network by the secretion inhibitor FLI-06. J Cell Sci 129: 3868-3877

3.3. DNA Damage Response In Neural Network And Postmitotic Neurons *(Wang)*

Genomic instability plays a vital role in cellular functionality and tissue homeostasis. The cell has evolved a sophisticated DNA damage response (DDR), including damage signaling, DNA repair, cell cycle control, apoptosis and transcription. We use cellular and molecular tools as well as animal models to dissect how the dysfunction of DDR affect neural cells and cause neurodevelopmental disorders and neuropathies. In the supporting period, we carried out genetic, cellular and molecular analyses of DNA damage response, governed by NBS1 and ATR using cellular and animal models. We also applied proteomic and metabolic studies to dissect the molecular pathways affected by the malfunctional DDR in neural cells including postmitotic neurons. Our research provides insights into aging-related pathogenesis, such as tissue decline and neurodegeneration. Our data identified novel functions of the classical DDR molecules in specific cell types, i.e. postmitotic cells, which have been overlooked when most studies using proliferating cellular systems. DDR molecules plays (perhaps more) important physiological roles in the network of developmental pathways in addition to its canonical DDR function, given the facts that our body is composed of most non-proliferating cells and that many DNA lesions may not occur or many DDR pathways may be muted in postmitotic cells.

3.3.1 DNA damage response in postmitotic neurons

DNA damage triggers two key DNA damage response (DDR) pathways mediated by ATM and ATR. The MRN (MRE11/RAD50/NBS1) complex functions mainly by activating ATM in response to DNA double strand breaks (DSBs) and also regulates the ATR activation in response to replication stress [1, 2]. Mutations of key components of the DDR lead to human chromosomal instability syndromes, in which neurological defects are in common [3]. For

example, mutations of ATM, ATR, NBS1 and MRE11 cause Ataxia-Telangiectasia (A-T), Seckel Syndrome, Nijmegen Breakage Syndrome (NBS) and A-T Like Disorder (A-TLD), respectively. A-T and A-TLD are associated with cerebellar degeneration, NBS and ATR-Seckel are characterized with microcephaly and intellectual disabilities. NBS1 and ATR are essential genes, because null mutation of either of them causes lethality to cells and animals.

We previously showed that a central nervous system (CNS)-specific deletion of Nbs1 in mice resulted in ataxia and a neuronal attrition that is Atm-p53 dependent [4]. Furthermore, we found that the Nbs1 deletion activates Hzf, which induces p53-mediated proliferation arrest of neuroprogenitors [5]. We also investigated whether these two molecules interact to prevent neuropathology. We found that whereas most apoptosis in the Nbs1-deleted cortex was restricted to the highly proliferating progenitors, the Atr knockout induced apoptosis in both proliferating and non-proliferating neural cells [6]. Thus, Nbs1 and Atr play a distinct role in neurogenesis, namely a specific function of Nbs1 in proliferating neuroprogenitors and of Atr in both proliferating and non-dividing cells.
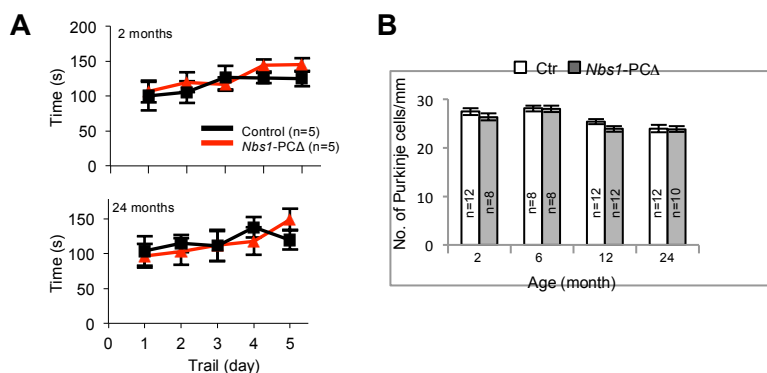

Fig 3.3.1. (A) Rotarod test reveals normal motor coordination of *Nbs1*-PCΔ mice at both 2 and 24 months of age. n = number of mice analyzed. (B) Purkinje cell density of control (Ctr) and *Nbs1*-PCΔ mice at indicated age. *n* = number of brain sections. Student *t*-test revealed no significant difference between both groups

To further investigate whether Nbs1-deletion mediated ataxia is specifically due to defects in Purkinje cells and to address whether Nbs1 has an essential function in postmitotic cell, we generated Purkinje cell-specific Nbs1 knockout mice (*Nbs1*-PCΔ). These mice developed and behaved normally during an observation period of 2 years (Fig 1A). The morphology and numbers of Purkinje cells of *Nbs1*-PCdel mice were apparently normal in the 2-year observation period (Fig 1B), despite both Mre11 and Rad50 were exclusively located in the cytoplasm of *Nbs1*-PCdel mutant Purkinje cells indicating a DDR defect (not shown). Thus Nbs1-mediated DDR is dispensable for the maintenance and functionality of postmitotic Purkinje cells.
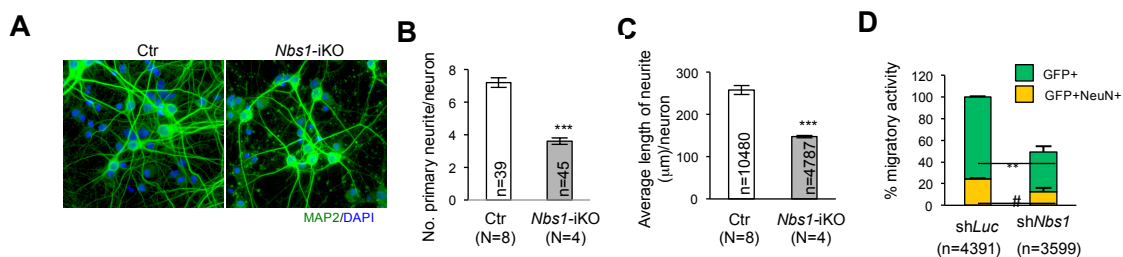

Fig 3.3.2. (A) Neurons without (Ctr) or with *Nbs1* inducible deletion (*Nbs1*-iKO) were stained with the dendritic marker MAP2. The number (B) and the average length (C) of dendrites per neuron were quantified from indicated number of neurons (n). N: the number of animals. (D) *Transwell* migration assay of primary cortical neurons from E17.5 after Nbs1 knockdown. The migratory activity was calculated as GFP+, or GFP+NeuN+ cells among the number of DAPI positive cells counted (n). Student *t*-test. #: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; n.s. = not significant.

However, when we deleted Nbs1 in primary neurons isolated from Nbs1/f/f-CER by tomoxifen mice (Nbs1-iKO), neuron arborization and migration were affected. Staining of the

culture with the neuron marker NeuN revealed a similar number of control and *Nbs1*-iKO neurons in cultures (not shown), suggesting no cell death without Nbs1. Surprisingly, the neurite complexity of neurons was evidently compromised after *Nbs1* deletion (Fig 2A). The number as well as the average length of primary neurites per neuron was significantly reduced in *Nbs1*-iKO neurons (Fig 2B, C). We next investigated the role of Nbs1 in neuronal migration by a *transwell* migration assay using GFP-sh*Nbs1*-knockdown in primary cortical neurons and found that sh*Nbs1*-transfected neurons had less migratory activity compared to sh*Luc* controls (Fig 2D).

It is well known that Notch signaling plays a critical role in neural development and morphogenesis of neurons [7, 8]. Consistent with this notion, we found that Nbs1 deletion led to up-regulation of the Notch1 RNA level as well as a high level of Notch1 intracellular domain (NICD1) in Nbs1 deleted neurons (Fig 3A, B). As expected, Notch1 activity was enhanced in these mutant neurons, which nevertheless could be repressed by the Notch1 inhibitor DAPT (Fig. 3C). We next investigated how Nbs1 could molecularly regulate Notch1. *Notch1* is a well-known auto-regulated gene [9, 10] and found that Nbs1 bound to promoter region of the *Notch1* gene (Fig. 3D). Furthermore, Co-IP experiments confirmed a direction interaction between NBS1 and NICD1 (Fig. 3E, F).
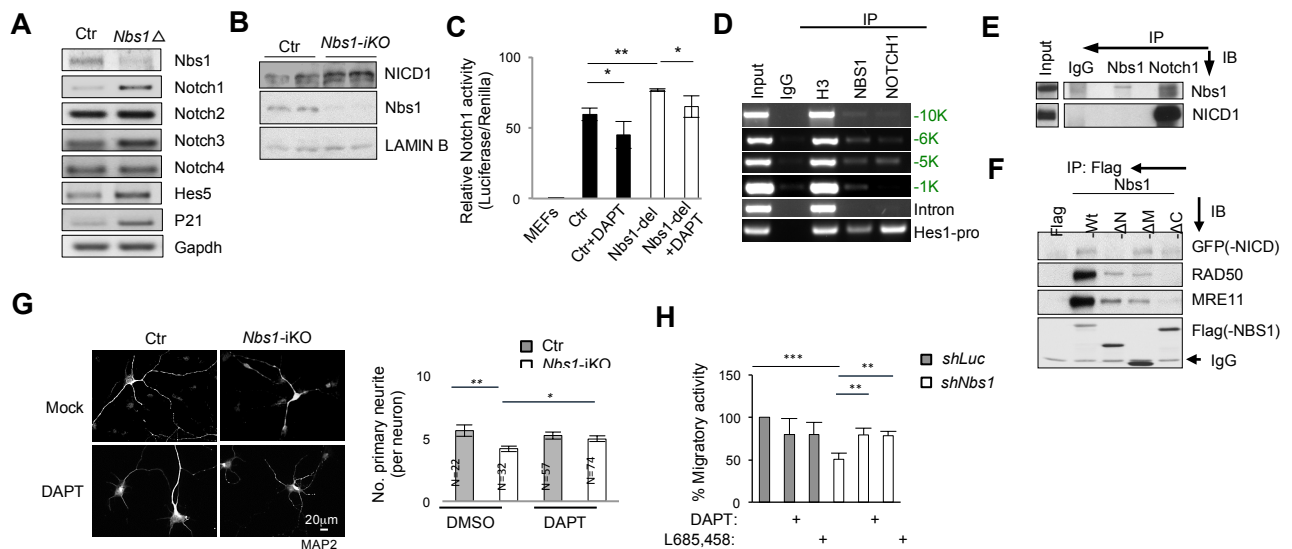

Fig. 3.3.3. (A) Semi-quantitative RT-PCR analysis of Notch receptors and its target genes, neurons isolated from cerebelli of *Nbs1*-CNSΔ and control mice. (B) Western blot analysis of *Nbs1*-iKO neurons with antibodies against Nbs1 and Notch1 that detects NICD1. Lamin B is a loading control. (C) Notch1 activity assay of *Nbs1*-iKO mouse embryonic fibroblasts (MEFs) treated with or without 5μM Notch inhibitor DAPT. Data were obtained from three independent experiments. (D) ChIP analysis of Nbs1 binding to the promoter regions of *Notch1* and its targets in MEF cells. PCR amplification of indicated Notch1 promoter/intron regions and the *Hes1* promoter was performed after ChIP with antibodies against IgG (negative control), Histone 3 (positive control), Nbs1 and Notch1. (E) Co-immunoprecipitation (Co-IP) analysis of endogenous interaction between Nbs1 and NICD1 in HeLa cells. (F) Identification of the binding domains between Nbs1 and Notch1 by Co-IP. The Notch1 antibody detects NICD1. HEK293 cells were co-transfected with Flag-tagged full length or truncated mutants of Nbs1 together with GFP-tagged NICD1. IP (IP:Flag) samples were subject for immunoblot analysis with indicated antibodies. (G) Notch inhibitor (10μM DAPT) rescues the arborization defects of *Nbs1*-iKO neurons (MAP2+). Quantification of the number of primary dendrites from each neuron (total n is indicated) is shown on the right. The number of neurons from at least two samples of each genotype was quantified. Data was from three independent experiments. (H) Rescue of the migration defect of *Nbs1* knockdown Neuro2A cells by Notch inhibitors (10μM of DAPT or L685, 458). Student *t*-test. *: p<0.05; **: p<0.01; ***: p<0.001.

To examine whether the abnormal activation of Notch1 is responsible for the morphology defect in the Nbs1 deleted neurons, differentiation was induced in N2A cells prior to treatment with Notch1 inhibitors (DAPT or L685,458) after transfection with shRNA to knock down Nbs1. Consistent with neurons, Nbs1 knockdown dramatically reduced the number of primary neurites in differentiated N2A cells (Fig. 3G). Notch1 inhibitors ameliorated the neurite defects in *Nbs1*-deficient N2A cells (Fig. 3G). To test whether the Notch1 pathway is involved

in the neuronal migration after Nbs1 deletion, we performed a *transwell* migration assay using Notch inhibitors in N2A cells after Nbs1 knockdown and found that Notch inhibitors rescued the migration activity of Nbs1 knockdown cells (shNbs1) reaching a level similar to controls (shLuc) (Fig. 3H). These data indicate that the defect of neurite outgrowth and neuronal migration in Nbs1 deficient neurons is due to a hyperactive Notch activity Taken all together, our study identify that DDR molecule NBS1 has a non-canonical and physiological function in postmitotic cells by regulating Notch1 signaling in neuronal maturation and migration.

3.3.2. The role of ATR in neuropathies and mitochondria functionality

To study the function of ATR in postmitotic neurons, we deleted ATR in Purkinje cells in Atr-PCΔ mice. Mutant mice showed motor dysfunction starting from 6-9 months of age (Fig 4A), which however had no sign of Purkinje cell loss and exhibited normal cerebellar morphology (Fig 4B, C), suggesting ATR is dispensable for Purkinje cell survival. Electrophysiology experiments revealed that *Atr*-knockout Purkinje cells have a higher firing activity and smaller interspike intervals (ISI) (Fig 4DE). The results indicate that ATR deletion causes a defective electrophysiology, particularly in transmitting inhibitory signals to spinal cord to coordinate locomotor actions. Moreover, ATR deletion in the glutamatergic excitatory neurons of the forebrain rendered ATR-FBΔ (deletion of ATR in forebrains) mutant mice susceptible to epileptic seizures after 8 months of age, which was associated with axonal and mossy fiber sprouting and astrogliosis in the dentate gyrus (Fig 5A, B). The electrophysiology of lateral performant pathway (LPP) synapses revealed that *Atr*-knockout hippocampal neurons had a higher action potential (AP) amplitude with a longer AP duration (Fig 5C, D). Interestingly, RNA-sequencing (not shown) and proteomics analysis of ATR-FBΔ mouse brains revealed annotations of neuropathies as well as mitochondrial dysfunctions (Fig 5E, F). Collectively, these results suggest a non-canonical function of ATR in the regulation of neuronal activity and synaptic plasticity.
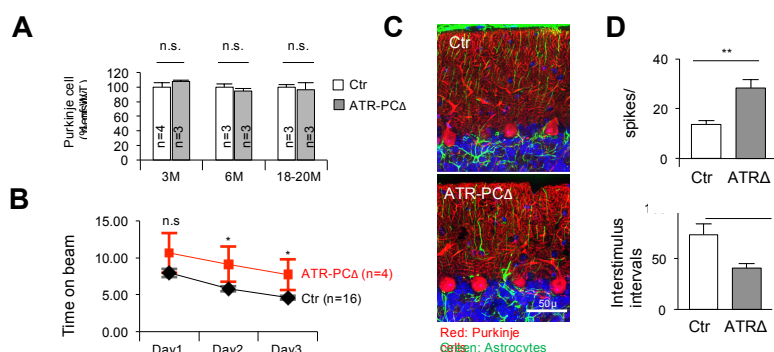

Fig 3.3.4. (A) Purkinje cell density of *Atr*-PCΔ mice at the indicated age. (B) A Rotarod test reveals motor coordination defect of *Atr*-PCΔ mice. n: number of mice. (C) Immunostaining of *Atr*-PCΔ cerebellum after staining with Calbindin (red, for Purkinje cells) and GFAP (green, for astrocytes). (D) Electrophysiological analysis of spike frequencies and intervals of spikes. Student's *t*-test. *: p<0.05; **: p<0.01; n.s. = not significant.
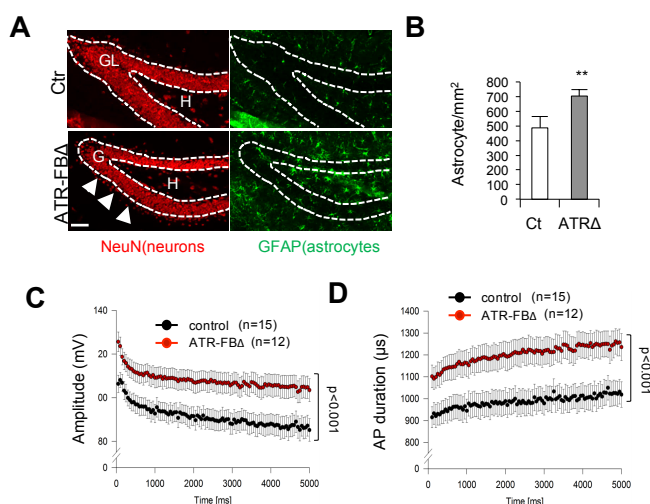
Fig 3.3.5. (A) Coronal brain sections of 12 months old of mice. NeuN and GFAP staining detect neuronal sprouting (arrowheads) and high GFAP signal (astrogliosis) in the dentate gyrus of *Atr*-FBΔ mice. GL: granular layer; H: hilus. (B) Quantification of GFAP+ astrocytes on the entire hippocampal area. Student's *t*-test. **p<0.01 (n=5). (C) Action potential amplitudes within the 20Hz AP training. *Atr*-FBΔ neurons show significantly increased AP amplitude. (D) Plot of half-height width of action potentials in whole-cell recorded dentate gyrus granule cells within the 20 Hz train stimulation of lateral perforant fibers. *Atr*-FBΔ neurons show significantly broadened AP. C and D: 2-way ANOVA with Holm-Sidak Post Hoc Analysis.

A
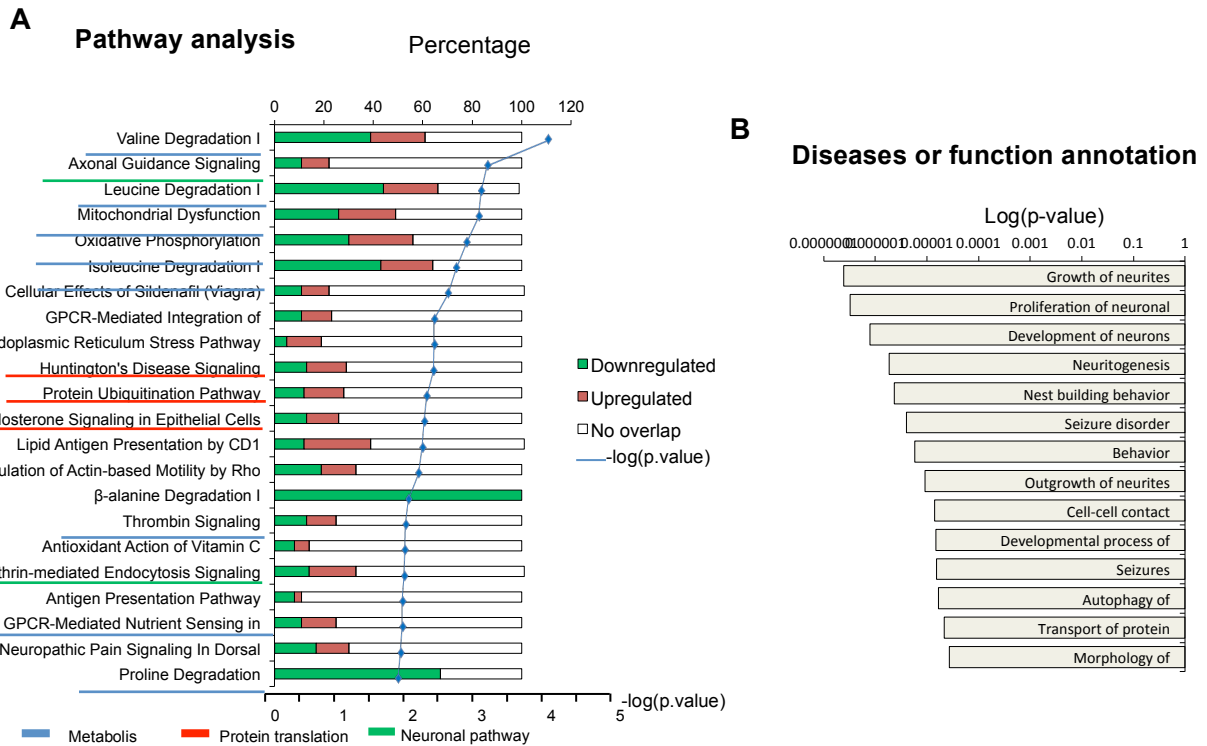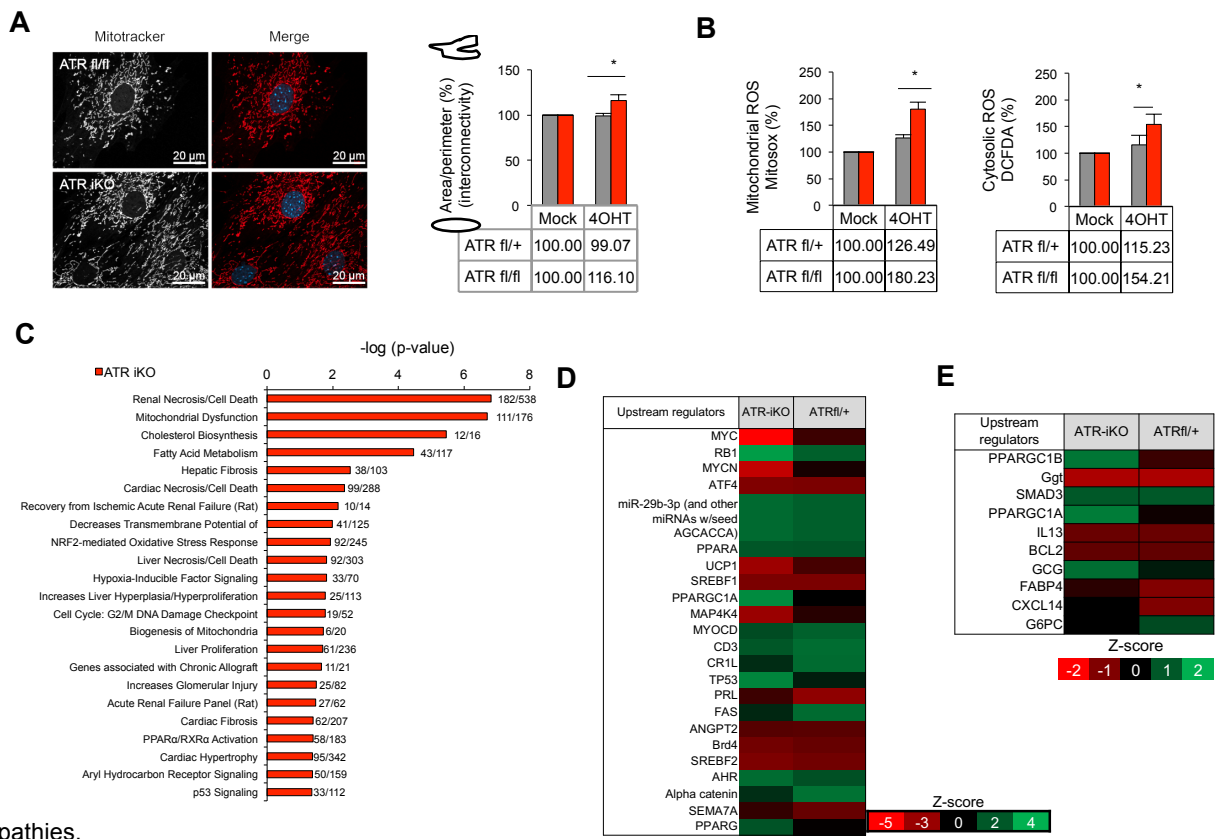
Pathway analysis

Percentage

Legend:
- ■ Downregulated
- ■ Upregulated
- □ No overlap
- — -log(p.value)

Categories:
- ▬ Metabolis
- ▬ Protein translation
- ▬ Neuronal pathway

B

Diseases or function annotation

Log(p-value)

Terms:
- Growth of neurites
- Proliferation of neuronal
- Development of neurons
- Neuritogenesis
- Nest building behavior
- Seizure disorder
- Behavior
- Outgrowth of neurites
- Cell-cell contact
- Developmental process of
- Seizures
- Autophagy of
- Transport of protein
- Morphology of

Fig 3.3.6. (A) Proteomics analysis of 3 months old control and *Atr*-FBΔ hippocampus (n=5) revealed mitochondrial dysfunctions (blue underline). Analysis is done with the IPA® software using 87 differentially expressed proteins (p<0.05). (B) Disease and function annotations of proteomics analysis show pathways involved in neuro-

A

	Mock	4OHT
ATR fl/+	100.00	99.07
ATR fl/fl	100.00	116.10

Area/perimeter (%) (interconnectivity)

B

Mitochondrial ROS Mitosox (%)

	Mock	4OHT
ATR fl/+	100.00	126.49
ATR fl/fl	100.00	180.23

Cytosolic ROS DCFDA (%)

	Mock	4OHT
ATR fl/+	100.00	115.23
ATR fl/fl	100.00	154.21

C

-log (p-value)

ATR iKO

D

E

pathies.

Fig 3.3.7. (A) Alterations of mitochondrial networks in ATR deleted pMEFs. Mitochondrial networks were evaluated using Mitotracker and analyzed by fluorescence microscopy in 200 (± 4OHT) ATR fl/fl and 100 (each ± 4OHT) ATR fl/+ cells from 4 independent experiments using ImageJ. Mitochondrial area/perimeter (interconnectivity) normalized to circularity was calculated. (B) Mitochondrial superoxide (via MitoSox) and cytosolic

15

peroxide (via DCFDA) levels in ATR fl/fl and ATR fl/+ pMEFs with or without 4OHT were analyzed by flow cytometry. Student's *t*-test. *: *p*<0.05. (C) Proteomic analysis of ATR deleted pMEFs. Proteomic (D) and metabolomic (E) analyses of signaling pathways in ATR deleted pMEFs. Pathway alterations are represented as sorted list depending on their Z-score. The color code defines the Z-score of individual signaling pathway. Pathway analysis by IPA® software.

To further study the role of ATR in mitochondrial function and metabolism, which may contribute to the neural phenotype of the patients and the mouse models, we used mouse embryonic fibroblasts (MEFs) isolated from *Atr*-CreER mice, in which ATR can be deleted in culture by 4-OHT. ATR deletion in primary mouse embryonic fibroblasts increased the connectivity of mitochondria, the level of reactive oxygen species (ROS), and the mitochondrial membrane potential and altered mitochondrial network structures (Fig 7A, B). Proteomics and metabolomics analyses revealed an imbalance of mitochondrial proteins and a dysfunction of the mitochondrial metabolism (Fig 7C), accompanied by altered metabolic signaling pathways, such as AMPK and PGC1α (Fig 7D, E). Consistently, we found high levels of oxidized proteins and an alteration of AMPK and PGC1α signaling pathways in the ATR-deleted embryonic brain (not shown).

Altogether, proteomic analyses in neural tissues and MEFs showed prominent alterations in mitochondrial functionality in ATR-deleted neurons. Thus, our studies indicate that ATR plays, in addition to its canonical DDR function, an important role for cell metabolism and mitochondrial functionality. These molecular changes in mitochondrial functionality and metabolic signaling can well explain the neuropathies associated with the neurological abnormalities of mutant animal models, as well as neuronal deficits of human Seckel syndrome.

3.3.4. Manuscripts

Submitted:

Zhong-Wei Zhou, Murat Kirtay, Nadine Schneble, George Yakoub, Tina Rüdiger, Kanstantsin Siniuk, Tang-Liang Li, Christoph Kaether, Ari Barzilai, Zhao-Qi Wang. Non-canonical function of NBS1 regulates maturation and migration of postmitotic neurons by modulating Notch1 signaling

In preparation:

Christian Marx et al. ATR regulates mitochondrial function and metabolism

Murat Kirtay et al. Biological function of ATR in epilepsy and neuron loss.

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4. Deliverables

As outlined above in the subprojects the SAW project improved our understanding of the role of DNA damage responses in driving tissue and organism aging. The project resulted in5 Master theses, 2 PhD theses, and 4 publication that have either been submitted or are in final stages of preparation for submission.

5. Data handling

All data were generated and archived strictly according to good scientific practice rules, which have been reinforced in the last 2 years at FLI. Results will be published as theses or paper contributions. According to newly implemented rules at FLI, computerized programs (external company) check all theses and papers for scientific integrity prior to submission. Omics data (such as RNAseq, proteomic anaylses etc.) are made publicly available on appropriate data-bases during the course of publication. The FLI supports the open access publications.