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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to in the text by Roman numerals (I-II). In addition, unpublished results are included. The original publications have been reproduced with permission of the copyright holders.

I **Antila C**, Rraklli V, Blomster H, Dahlström K, Salminen T, Holmberg J, Sistonen L, Sahlgren C. Sumoylation of Notch1 represses its target gene expression during cell stress. *Cell Death Differ* 25:600-615, 2018

II Lähdeniemi I, Misiorek J*, **Antila C***, Landor S, Stenvall C, Fortelius L, Bergström L, Sahlgren C[#], Toivola D[#]. Keratins regulate colonic epithelial cell differentiation through the Notch1 signaling pathway. *Cell Death Differ* 24:984-996, 2017

III Antfolk D, **Antila C**, Kemppainen K, Landor S, Sahlgren C. Decoding the PTM-switchboard of Notch. *Biochim Biophys Acta Mol Cell Res*. 1866:118597, 2019

* Equal contribution
[#] Shared corresponding authorship

ABBREVIATIONS

ADAM	A disintegrin and metalloproteinases
ALL	Acute lymphoblastic leukemia
APP	Amyloid precursor protein
ATL	Adult T-cell leukemia
B-ALL	B-cell acute lymphoblastic leukemia
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CIR	CBF1 interacting corepressor
CLL	Chronic lymphocytic leukemia
DUB	Deubiquitinase
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FANCD2	Fanconi's anaemia complementation group D2
FBXW7/SEL-10	F-box/WD40 domain-containing protein
FLN	Full length Notch
GFP	Green fluorescent protein
GSI	γ-secretase inhibitors
HD	Heterodimerization domain
HDAC	Histone deacetylase
HERP	HES-related repressor protein, most often referred to as Hey
HES	Hairy and enhancer of split
HIF-1α	Hypoxia-inducible factor 1 alpha
IBD	Inflammatory bowel disease
MAML	Mastermind-like
MDM2	Murine double minute 2
NRR	Negative regulatory region
NICD	Notch intracellular domain
PCNA	Proliferating-cell nuclear antigen
RanGAP1	Ran-GTPase-activating protein
RPB-J	Recombining binding protein suppressor of hairless
RITA	RBP-J interacting and tubulin associated
RNAi	RNA interference
SCD	Spondylocostal dysostosis
SEK	Simple epithelial keratin
SENP	Sentrin-specific protease
SMRT	Silencing mediator of retinoid and thyroid hormone receptor
SRA	RNA steroid receptor coactivator
SUMO	Small ubiquitin-like modifier
TA	Transit amplifying
T-ALL	T-cell acute lymphoblastic leukemia
UBL	Ubiqitin-like protein
UBR5	Ubiquitin protein ligase E3 component n-recognin 5
USP5	Ubiquitin carboxyl-terminal hydrolase 5
VEGF	Vascular endothelial growth factor

ABSTRACT

The Notch signaling mechanism is a crucial regulator of stem cells during development and tissue regeneration. Transcriptional activation of Notch target genes requires cleavage of the Notch receptor in response to ligand binding, production of the Notch intracellular domain (NICD), NICD migration into the nucleus, and assembly of a transcriptional complex. This thesis focuses on the stress-induced interaction of Notch with the small ubiquitin-like modifier (SUMO) and the effect of this interaction on the differentiation of neuronal stem cells in the central nervous system (Study I), and the interaction of Notch with the cytoskeletal intermediate filament (IF) keratin proteins in the differentiation of intestinal epithelial cells (Study II). Study (III) is a review and focuses on discussing different aspects of various post-translational modifications (PTM) of Notch.

The Notch receptor interacts with several post-translational modifiers, but the exact mechanisms and consequences of these interactions remain elusive. PTMs of Notch regulate its trafficking, turnover, and transcriptional activity. Study (I) presents a direct interaction between Notch and SUMO during cell stress in the form of heat shock or the presence of the proteasome inhibitor termed bortezomib. The sumoylation took place in the nucleus where SUMO was conjugated to the transcription factor Suppressor of Hairless (RBPJ/CSL)-associated domain (RAM) of NICD. In canonical Notch signaling, the interaction between RAM and the transcription factor Recombination signal binding protein for immunoglobulin kappa J region (RBPJ)/CSL is a prerequisite for activation of Notch target genes. However, the sumoylation site in the RAM domain was easily accessible for sumoylation without affecting the RAM-CSL interaction or any other component within the DNA-bound transcriptional complex consisting of NICD, CSL and the coactivator Mastermind-like (MAML). Neither did the SUMO-conjugating protein, termed ubiquitin conjugating enzyme 9 (Ubc9) interfere in the NICD-CSL-DNA interactions. Therefore, NICD sumoylation occurred in the transcriptional complex without disrupting the complex. Cellular stress-induced sumoylation of NICD enhanced the protein level and nuclear localization of Notch, but nevertheless still dampened its transcriptional activity. On a mechanistic level, NICD sumoylation promoted the recruitment of histone deacetylase 4 (HDAC4) to the transcriptional complex to suppress Notch target gene expression. The NICD sumoylation-mediated repression of Notch target genes was abolished by expression of a sumoylation-deficient mutant Notch construct in cells *in vitro*. Furthermore, the stress-inducible sumoylation provided a regulatory mechanism for dynamic Notch target gene expression also *in vivo*. In the developing central nervous system of chicken embryos, the expression of Notch target genes was repressed upon cellular stress by wildtype Notch, whereas the target gene activation capacity was retained by a sumoylation-deficient Notch mutant. In addition, consequent differences between the wildtype and the sumoylation-deficient Notch regarding tissue differentiation were displayed. The findings of the stress-inducible sumoylation of NICD in study (I), revealed a novel context-dependent regulatory mechanism of Notch target gene expression.

Study (II) of this thesis presents a direct interaction between Notch and keratins and the outcomes that this specific interaction mediates on the proliferation and differentiation of colonic epithelial cells. Keratins are cytoskeletal intermediate filament (IF) proteins and have essential roles in cellular stress protection and mechanical support of epithelial tissues.

K8, K18 and K19 constitute the largest group of the keratin isoforms that are expressed in the colon. K8 is along with K18 the main component, not only of all keratin isoforms, but also of the entire IF cytoskeleton and is therefore also the main IF protein of simple layered epithelia. K8 is localized preferentially in the cytoplasm and is the most common type II keratin, pairs predominantly with the type I K18, and is mainly expressed in the epithelia of the intestine, liver and exocrine pancreas. Earlier it has been known that mice lacking K8 ($K8^{-/-}$ mice) develop colitis, decreased apoptosis and significant hyperproliferation of colonic crypt epithelial cells, but the effect of the loss of K8 on intestinal cell differentiation had so far been unknown. Notch in turn has been known to be a critical regulator of colonic epithelial cell differentiation. In study (II), we showed that K8 together with its polymeric partner K18 regulated Notch signaling activity and cell differentiation in the epithelium of the large intestine. K8 bound directly to Notch1 in colonic epithelial cells, both *in vitro* in cell culture, and *in vivo*, displayed in immunoprecipitations and proximity ligation assays. *In vitro*, the interaction resulted in enhanced Notch levels and activity in a dose-dependent manner, but unlike the Notch-SUMO interaction discovered in study (I), which resulted in repressed Notch target gene expression, the Notch-K8/K18 interaction in study (II) led to increased expression of specific Notch target genes. Accordingly, *in vivo* in the colon epithelium, $K8^{-/-}$ mice had decreased levels of both the complete form of Notch, called full length Notch (FLN), and NICD, and also repressed expression of the Notch target genes Hey1 and Hey2. The effect of the absence of K8 on Notch activity was observed in cultured colorectal cancer cells by deleting K8 with CRISPR/Cas9. This knockout of K8 led to the downregulation of FLN and NICD levels and reduced Notch downstream target gene Hey1 expression, which was rescued with re-expression of K8/K18. *In vivo*, the differentiation phenotype in $K8^{-/-}$ colonic epithelium was shifted from an enterocyte cell fate towards increased goblet cells and enteroendocrine cells. Moreover, in the colonic crypts, K8 depleted mice developed increased amount of transit amplifying (TA) cells from which the terminally differentiated cells originate. In study (II), Notch interacted with K8/K18 and regulated Notch signaling during differentiation in the colonic epithelium.

TYÖN TIIVISTELMÄ

Notch-reseptori on elintärkeä kantasolujen säätelijäproteiini kudosten kehitysvaiheessa ja uusiutumisessa. Notchin kohdegeenien aktivointi edellyttää Notch-reseptorin pilkkoutumista Notchin aktivoinnin jälkeen, tuottaen NICD-proteiinin (eng. Notch intracellular domain), joka siirtyy solun tumaan muodostaen Notchin kohdegeenejä aktivoivan proteiinikompleksin. Tämä väitöskirja keskittyy selventämään Notch-SUMO (eng. small ubiqutin-like modifier)-vuorovaikutusta, ja Notchin vuorovaikutusta solun tukirangan sytoskeletonin sisältämien intermediaarifilamentti (IF)-proteiinien keratiinien kanssa, ja kyseisten vaikutussuhteiden seuraamuksia solukohtalolle ja kudossolujen erikoistumiselle solustressin aikana keskushermostossa (Projekti I) ja paksusuolen epiteelisolujen erikoistumisen aikana (Projekti II). Projekti (III) on katselmus, jossa käydään läpi miten eri tunnetut Notchin posttranslationaaliset modifikaatiot vaikuttavat Notch-signaalimekanismin eri osa-alueisiin.

Notch-reseptoriproteiini on vuorovaikutuksessa monen posttranslationaalisia modifikaatioita suorittavan proteiinin kanssa, mutta kyseisten vuorovaikutusten tarkat mekanismit ja seuraamukset ovat epäselviä. Notchin posttranslationaaliset modifikaatiot säätelevät Notchin sijaintia, hajoamista ja Notchin kohdegeenien aktiviteettia. Projekti (I) esittelee suoran vuorovaikutuksen Notchin ja SUMO:n välillä, jonka aiheuttaa erilaiset solustressit kuten esimerkiksi lämpöshokki, ja proteasomi-inhibiittori nimeltään bortezomib. Notchin sumolaatio tapahtui solun ytimessä, jossa NICD sumoloitui RAM (eng. transcription factor suppressor of Hairless (RBPJ/CSL)-associated domain)-domeenissaan. Useimmiten Notch-signaalimekanismissa RAM-domeenin vuorovaikutus CSL (eng. Recombination signal binding protein for immunoglobulin kappa J region (RBPJ))-proteiinin kanssa on edellytys Notchin kohdegeenien aktivoinnille. Notchin RAM-domeenin sumolaatiokohta oli kuitenkin helppopääsyinen SUMO:lle ilman että sumolaatio vaikutti RAM-CSL vuorovaikutukseen tai mihinkään muuhunkaan osaan DNA:han kiinnittyneessä Notchin kohdegeenejä aktivoivassa proteiinikompleksissa, joka sisältää muun muassa NICD-, CSL- ja MAML (eng. Mastermind-like)-proteiinit. Myöskään SUMO:n kiinnittävä Ubc9 (eng. Ubiquitin conjugating enzyme 9)-proteiini ei vaikuttanut NICD-CSL-DNA välisiin vuorovaikutuksiin. Täten NICD:n sumolaatio tapahtui proteiinikompleksissa ilman kyseisen kompleksin muodostumisen tai ylläpitämisen häiritsemistä. Solustressin aikaansaama NICD:n sumolaatio lisäsi NICD:n määrää, mutta vähensi silti NICD:n kykyä aktivoida kohdegeenejään. Mekanistisella tasolla NICD:n sumolaatio tehosti HDAC4 (eng. Histone deacetylase 4)-proteiinin rekrytointia proteiinikompleksille vähentäen Notchin kohdegeenien aktivointia. NICD:n sumolaation välittämä Notchin kohdegeenien vaimennus lakkasi sumolaatioon kykenemättömän NICD-mutanttikonstruktin tuotannosta *in vitro* (elävän organismin ulkuopuolella, kuten lasimaljassa tehty tutkimus), mutta solustressin aikaansaama sumolaatio toimi säätelevänä mekanismina dynaamiselle Notchin kohdegeenien aktivoinnille myös *in vivo* (elävässä organismissa tehty tutkimus, kuten hiirikokeet). *In vivo* kanan alkion kehittyvässä keskushermostossa, Notchin kohdegeenien aktivointi vaimentui solustressin aikaansaaman villityyppi-NICD:n sumolaation toimesta, kun sitä vastoin kohdegeenien aktivointi säilyi entisellään sumolaatioon kykenemättömän NICD-mutanttikonstruktin toimesta. Tämän lisäksi esiintyi seuraamuksellisia eroja villityyppi-Notchin ja mutantti-Notchin välillä kudosten erikoistumisessa. Projekti (I) esittelee

solustressin aikaansaaman NICD:n sumolaation uutena Notchin kohdegeenien aktivointia säätelevänä mekanismina, jonka vaikutukset ovat kudostyypistä, ympäristöstä ja olosuhteista riippuvaisia.

Tämän väitöskirjan projektissa (II) esitellään suora vuorovaikutus Notchin ja keratiinien välillä, ja seuraamuksia, joita nämä vuorovaikutukset välittävät paksusuolen epiteelikantasolujen jakautumiselle ja erikoistumiselle. Keratiineilla on oleellisia tehtäviä sekä solun suojelemisessa solustressiltä että epiteelikudosten mekaanisessa ylläpidossa. K8, K18 ja K19 ovat yleisimmät paksusuolessa esiintyvät keratiiniproteiinit. K8 ja K18 ovat, eivät ainoastaan pääasialliset keratiinit, vaan myös koko sytoskeletonin pääasialliset IF-proteiinit, ja ovat täten pääasialliset IF-proteiinit myös yksinkertaisessa / yksikerroksisessa epiteelikudoksessa. K8 ilmentyy pääasiallisesti sytoplasmassa (solulimassa) ja on yleisin II-tyypin keratiini, muodostaa proteiinipareja tyypin I K18-proteiinin kanssa ja syntetisoituu lähinnä epiteelikudoksissa suolistossa, maksassa ja haimassa. Ennen tämän väitöskirjan tutkimuksia on tiedetty, että K8-puutteellisilla hiirillä ($K8^{-/-}$-hiirillä) kehittyy paksusuolessa esiintyvää tulehdusta, vähentynyttä ohjelmoitua solukuolemaa ja haitallisen voimakasta jakautumista paksusuolen kryptojen epiteelisoluissa, mutta K8-proteiinin puutteen vaikutus suoliston kantasolujen erikoistumiseen oli ollut tuntematon. Notchin sitä vastoin on aiemmin tiedetty olevan kriittisen tärkeä säätelijä paksusuolen epiteelikantasolujen erikostumisessa. Tämän väitöskirjan projekti (II) näyttää toteen, että K8 yhdessä K18-proteiinin kanssa säätelee Notch-signaalimekanismia ja paksusuolen epiteelikudoksen kantasolujen erikoistumista. K8 kiinnittyi suoraan Notchiin paksusuolen epiteelisoluissa, sekä *in vitro* soluviljelmissä että *in vivo* hiirikokeissa. *In vitro*, Notch-K8/K18 vuorovaikutus johti kohonneisiin Notchin määriin ja aktiivisuuteen, jotka heijastuivat suoraan vuorovaikutuksen määrästä ja tehosta. Mutta päinvastoin kuin projektin (I) Notch-SUMO vuorovaikutus, joka johti Notchin kohdegeenien vähentyneeseen aktivointiin, Notch-K8/K18 vuorovaikutus projektissa (II) johti Notchin kohdegeenien lisääntyneeseen aktivointiin. Vastaavasti *in vivo*, paksusuolen epiteelissä $K8^{-/-}$-hiiret tuottivat vähentyneissä määrin sekä Notchin kokopitkää proteiinimuotoa nimeltään FLN (eng. Full length Notch) että NICD-proteiinia, ja ilmensivät myös Notchin kohdegeenien Hey1 ja Hey2 vähentynyttä aktivointia. K8-proteiinin poissoaolon vaikutuksen Notchin aktiivisuuteen havaittiin viljellyissä paksusuolen syövän soluissa poistamalla K8-proteiinit CRISPR/Cas9-tekniikalla. Tämä K8-proteiinin poistaminen johti FLN:n ja NICD:n vähentyneisiin määriin ja Notchin kohdegeenin Hey1 vähentyneeseen aktivointiin. Tämän Hey1-geenin vähentyneen aktivoinnin pystyi puolestaan kumoamaan tuottamalla uudestaan K8/K18-proteiinia. *In vivo*, solujen erikoistumiseen liittyvä fenotyyppi (kehittyvän tai kehittyneen yksilön havaittavien ominaisuuksien kokonaisuus) $K8^{-/-}$-hiirillä siirtyi paksusuolessa enterosyyttisestä solukohtalosta kohti lisääntynyttä gobletsolujen ja enteroendokriinisten solujen määrää. Tämän lisäksi, $K8^{-/-}$-hiirten paksusuolen kryptoissa esiintyi lisääntynyttä määrää TA (eng. transit amplifying)-soluja (solumuoto kantasolujen ja jo erikoistuneiden solujen välillä), joista lopullisesti erikoistuneet solut ovat erikoistuneet. Projektissa (II), Notch vuorovaikutti K8/K18-proteiinikaksikon kanssa ja säätelee Notch-signaalimekanismia solujen erikoistumisen aikana paksusuolen epiteelikudoksessa.

ABSTRAKT

Notch-signalmekanismen är en kritisk reglerare av stamceller under utveckling och förnyelse av vävnad. Den transkriptionella aktiveringen av Notch-målgener kräver klyvning av Notch-receptorn som respons till ligand-bindning, produktion av NICD (den intracellulära domänen av Notch), migration av NICD till cellkärnan, och sammanställning av ett transkriptionellt komplex. Denna avhandling fokuserar på Notch-SUMO (eng. small ubiqutin-like modifier)-växelverkan, och Notch-proteinets växelverkan med cellens cytoskeletala intermediärfilament (IF)-proteiner keratiner, och dessa interaktioners konsekvenser för cellödet och vävnadsdifferentiering under cellstress i centrala nervsystemet (CNS) (Projekt I) och under differentiering i tjocktarmen (Projekt II). Projekt (III) är en granskning som diskuterar hur olika posttranslationella modifieringar av Notch påverkar olika aspekter av Notch-signalmekanismen.

Notch-receptorn interagerar med flera post-translationella modifierare, men de exakta mekanismerna och konsekvenserna av dessa interaktioner är oklara. Post-translationella modifikationer av Notch reglerar dess translokalisering, omsättning och transkriptionella aktivitet. Projekt (I) presenterar en direkt interaktion mellan Notch och SUMO under cellstress i form av värmechock eller i närvaro av proteasom-inhibitorn bortezomib. Sumoyleringen skedde i cellkärnan där SUMO konjugerades till NICD i RAM (eng. transcription factor suppressor of Hairless (RBPJ/CSL)-associated domain)-domänen. I kanonisk Notch-signalering är RAM-CSL (eng. Recombination signal binding protein for immunoglobulin kappa J region (RBPJ)) interaktionen en förutsättning för aktivering av Notch-målgener. Sumoyleringsstället i RAM-domänen var enkelt tillgänglig för sumoylering utan att sumoyleringen påverkade RAM-CSL interaktionen eller någon annan komponent inom det DNA-bundna transkriptionella komplexet som består av NICD, CSL och MAML (eng. Mastermind-like)-proteinerna. Därutöver påverkade inte det SUMO-konjugerande proteinet Ubc9 (eng. Ubiquitin conjugating enzyme 9) NICD-CSL-DNA interaktionerna. Därmed kunde sumoyleringen av NICD ske utan att det transkriptionella komplexet förstördes. Cellstress-inducerad sumoylering av NICD ökade den nukleära lokaliseringen och nivåerna av Notch, men dämpade ändå dess transkriptionella aktivitet. På en mekanistisk nivå ökade sumoylering av Notch rekryteringen av HDAC4 (eng. Histone deacetylase 4) till det transkriptionella komplexet för att dämpa Notch-målgenernas uttryck. Den SUMO-medierade uttrycksrepressionen av Notch-målgener avskaffades vid uttryck av ett sumoyleringsbristfälligt Notch-mutantkonstrukt i celler *in vitro*, men stressinducerad Notch-sumoylering försåg en reglerande mekanism för dynamiskt uttryck av Notch-målgener också *in vivo*. *In vivo* i det utvecklande centrala nervsystemet av kycklingsembryon, dämpades uttrycket av Notch-målgener under cellulär stress av en vildtyp-Notch medan genaktiveringskapaciteten upprätthölls av en sumoyleringsbristfällig Notch-mutant. Därutöver detekterades olikheter i vävnadsdifferentiering mellan vildtyp-Notch och den sumoyleringsbristfälliga Notch. Forskningsresultaten i projekt (I) om stressinducerad sumoylering av NICD avslöjar en ny kontextberoende reglerande mekanism av Notch-målgenernas uttryck.

I projekt (II) av denna avhandling presenteras en direkt interaktion mellan Notch och keratiner och konsekvenser som denna specifika interaktion medierar för proliferationen och differentieringen av tjocktarmens epitelceller. Keratinerna är cytoskeletala IF-proteiner

och har essentiella roller i skydd mot cellulär stress, och också i mekaniskt upprätthållande av epitelvävnader. K8, K18 och K19 är de främst förekommande keratinisoformerna som uttrycks i kolonet. K8 och K18 är också de huvudsakliga IF-proteinerna i simpel epitelvävnad som består av endast ett epitellager. K8 lokaliseras företrädesvis i cytoplasmat och är det allmännaste typ II-keratinet, bildar heterodimerer främst med K18, och uttrycks mestadels i tarmen, levern och bukspottskörteln. Innan avhandlingens forskningar visste man att möss som saknar K8-proteinet ($K8^{-/-}$-möss) utvecklar kolit, minskad apoptos och signifikant hyperproliferation av epitelceller i kolons kryptor, men effekten K8-förlusten på tarmcellers differentiering hade hittils varit okänd. Notch i sin tur har varit känd för att vara en viktig reglerare av epitelcellers differentiering i kolon. Projekt (II) av denna avhandling visar att K8 tillsammans med sitt polymeriska partnerprotein K18 reglerar Notch-signalmekanismens aktivitet, och celldifferentieringen i kolons epitelvävnad. K8 band direkt till Notch i kolons epitelceller, både *in vitro* i cellkultur, och *in vivo* i experiment utförda med möss. *In vitro* ledde interaktionen till ökade nivåer och ökad aktivitet av Notch på ett dosberoende sätt, men i motsats till Notch-SUMO interaktionen i projekt (I), vilket ledde till dämpat uttryck av Notch-målgener, ledde Notch-K8/K18 interaktionen i projekt (II) till ökat uttryck av specifika Notch-målgener. Följdaktligen, *in vivo* i kolons epitelvävnad hade $K8^{-/-}$-möss sänkta nivåer av både den fullständiga formen av Notch kallad för FLN (eng. Full length Notch), och NICD, och nersänkt uttryck av Notch-målgenerna Hey1 och Hey2. K8-proteinets frånvaroeffekt på Notch-aktiviteten observerades i odlade koloncancerceller genom att radera K8 med tekniken CRISPR/Cas9. Denna radering av K8 ledde till nedreglering av FLN- och NICD-nivåer och nersänkt uttryck av Notch-målgenen Hey1, vilket kunde räddas med att återigen syntetisera K8/K18. *In vivo* var kolons differentieringsfenotyp i $K8^{-/-}$-möss flyttad från ett enterocytiskt cellöde mot ökade mängder av gobletceller och enteroendokrina celler. Därutöver, i kolons kryptor utvecklade $K8^{-/-}$-möss ökade mängder av TA (eng. transit amplifying)-celler från vilka de terminalt differentierade cellerna härstammar. I projekt (II) interagerade Notch med K8/K18 och reglerar Notch-signalmekanismen under differentiering i kolons epitelvävnad.

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INTRODUCTION

The extrinsic and intrinsic environment of the cell is characterized by strictly separated and delimited stimuli which integrate for the benefit of the organism. To maintain cellular tissue homeostasis, different forms of enzymatic activities fine-tuning cellular functions are required. This includes fine-tuning receptor-mediated signaling mechanisms between cells and post-translational modifications that regulate the activity of these communicators. Transmembrane receptors such as Notch function as integrators in these situations. The receptors receive specific signals that change the fate of the cell. The signals are mediated when an extracellular ligand such as a hormone, neurotransmitter, pharmaceutical agent, peptide or some other molecule binds to the receptor. Complexes formed between the ligand and the receptor most often lead to alterations in the intracellular setting. The Notch signaling pathway is initiated in response to ligand binding with consequent cleavage of Notch resulting in the translocation of the Notch intracellular domain (NICD) into the nucleus, and assembly of a Notch transcriptional complex leading to activation of Notch target genes.

The Notch receptor protein is a key regulator of stem cell function and aberrant activity is linked to developmental defects, genetic diseases and different forms of cancers. In terms of disease pathogenesis, Notch is known particularly for its role in the development of T-cell acute lymphoblastic leukemia (T-ALL), and solid cancers such as breast cancer. Cell-cell contact-dependent signaling via Notch is a biological process that regulates tissue development and repair, and is necessary for pre- and post- embryonic development in all multicellular organisms.

Regulation of cellular functions occurs partly through reversible processes that are essential in order to maintain homeostasis. Among these cellular functions exist rapidly occurring PTMs (Lee et al. 2015). These modifications regulate the function and state of their protein substrates and are a prerequisite to meet the changing challenges that cells continuously face. Ubiquitin-like modifications are involved one way or another in almost every cellular process. The PTM by small ubiquitin-like modifier (SUMO) proteins has since its discovery in the 1990's emerged as a critical regulatory mechanism of various cellular outcomes. The outcomes that sumoylation mediates are case-specific, but often involves e.g. alterations in the cellular localization of the target protein and alterations in gene transcription levels. Sumoylation is strictly controlled by the enzymes that participate in the SUMO conjugation machinery with the aim to either dampen or increase the efficiency of the modification (Pichler et al. 2017). In this thesis, project (I) elucidated the interaction between the Notch receptor and the SUMO protein, and consequences thereof for Notch in its pathway and concurrently for its target gene expression.

Another new Notch interacting protein partner affecting cellular outcomes are keratins, or more specifically the heterodimer K8/K18. The most important task of the cytoskeleton is to maintain and support the shape and motility of cells. Keratins are cytoskeletal proteins that are primarily expressed in epithelial tissue and make up the largest subgroup of intermediate filament proteins (Jacob et al. 2018). K8, K18 and K19 are the major epithelial keratin proteins in the colon and are regulated by several keratin-associated proteins. Project (II) of this thesis focuses on presenting the effect of the Notch-Keratin interaction

on the Notch signaling pathway and consequently on the proliferation and differentiation of colonic epithelial cells.

Both K8/K18 (Study II) and SUMO (Study I) mediate part of their cellular outcomes through protein-protein interactions with Notch. By binding directly to Notch, keratins and SUMO become protein members of Notch-driven tissue-specific cell fate.

Context-dependency is a major part of the characteristics of the Notch receptor. This thesis shows that both SUMO and keratins are regulators of the Notch signaling pathway. They both interact directly with Notch, and their behavior is impacted by a common nominator: cellular stress. A common feature in sumoylation is the conjugation of SUMO to a target protein as a consequence of cellular stress. Cell stress strongly affects the binding of SUMO to Notch. This does not only apply to Notch, but to a greater number of SUMO substrates. Keratins, on the other hand, are essential for epithelial stress protection, which is exemplified when keratins form heterodimers and form filaments which provide structural integrity of epithelia and mechanical resilience against cell stress. Therefore, keratins are generally considered in an increasing manner as stress proteins that protect cells and tissues from cell stress and injury. In addition, intestinal keratins are upregulated and undergo post-translational modifications, phosphorylation in particular, during cellular stress and recovery. Keratin mutations are linked to skin diseases such as Dowling-Meara epidermolysis bullosa simplex (EBS), and colon diseases such as colorectal carcinoma. Cellular stress thereby regulates both the conjugation/deconjugation of SUMO, and the activity of keratins. However, all in all, this thesis focuses more prevalently specifically on regulatory aspects and mechanisms of the Notch signaling pathway rather than the physiology affected by Notch via sumoylation or keratins.

REVIEW OF THE LITERATURE

1. THE NOTCH SIGNALING PATHWAY

Over a century ago, in 1914, mysterious notches were discovered in the wings of the fruit fly *Drosophila melanogaster* (Dexter 1914). Three years later the mystery was resolved when a phenotype characterized by the serrated (notched) ends in the wings was associated to a gene called Notch (Morgan 1917). However, the gene was not identified until 1985 when it was successfully sequenced (Wharton et al. 1985; Kidd et al. 1986).

1.1 The Notch family

The Notch gene encodes for a transmembrane glycoprotein receptor which exists in most eukaryotes (Artavanis-Tsakonas et al. 1999; Chari and Winandy 2008). All Notch receptors are composed of an extracellular domain, a small transmembrane domain and an intracellular domain which functions as a nuclear effector (Palaga et al. 2003). Four different human Notch isoforms exist: Notch1 (273 kDa), Notch2 (265 kDa), Notch3 (244 kDa) and Notch4 (210 kDa). The Notch isoforms possess great structural similarities (Gordon et al. 2008), but the functional regions vary depending on isoform and organism (Figure 1).

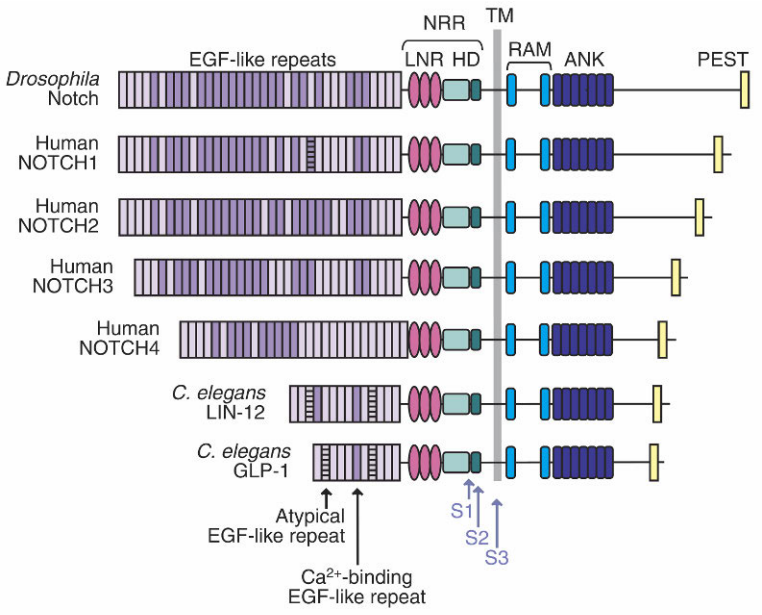

Figure 1. Schematic representation of the structure of the Notch receptor. The N-terminal domain of Notch consists of a group of EGF-like repeats. The human Notch receptors contain 29-36 EGF-like repeats while the Notch receptors of the nematode *Caenorhabditis elegans* comprise only 14 and 11 EGF-like repeats respectively. Notch intracellular domain (NICD) includes several functional regions including an N-terminal recombination binding protein-Jκ-associated molecule (RAM)-domain which in the canonical Notch signaling is required for binding to the transcription factor Recombination signal binding protein for immunoglobulin kappa J region (RBP-J)/CSL. NICD also includes an ankyrin repeat (ANK) domain, and a C-terminal PEST sequence which regulates the turnover of the pathway. For closer details of the mentioned regions, please see

section “1.2. Proteolytic activation of Notch”, and “1.3 Transcriptional regulation of Notch target genes”. The figure is adapted from (Gordon et al. 2008).

Signaling by Notch is a highly conserved signaling pathway in eukaryotes (Chiba 2006). It is believed that the fundamental features of Notch signaling evolved already during the Precambrian era 0.5-4.5 billion years ago (Hurlbut et al. 2007). The signaling mechanism thereby seems to be firmly evolutionarily conserved both in vertebrates and invertebrates including *Drosophila melanogaster* (Kiyota and Kinoshita 2002; Vasyutina et al. 2007).

The activity of Notch is regulated directly by interaction with a Notch ligand expressing cell (D’Souza et al. 2008; Funahashi et al. 2008). After specific ligand binding, the Notch receptor is cleaved extracellularly leading to deletion of the extracellular part of Notch. The remained membrane-associated part of Notch is called ΔE Notch (ΔEN) which is also quickly cleaved giving rise to Notch intracellular domain (NICD). NICD is translocated to the cell nucleus where it forms a transcriptional activation complex bound to the DNA, with Recombination signal binding protein for immunoglobulin kappa J region (RBPJ), also called CSL, and with Mastermind-like (MAML) resulting in activation of Notch target genes (Borggrefe and Oswald 2009). Signaling via Notch is an unusual way for the cell to mediate extracellular signals since the activity of Notch and the amplification of the signal are not dependent on secondary messengers (Tien et al. 2009).

1.2 Proteolytic activation of Notch

The extracellular domain of Notch undergoes glycosylation in the ER and Golgi apparatus prior to presentation on the cell membrane. In the trans-Golgi, Notch undergoes proteolysis at site 1 (S1) (Hurlbut et al. 2007). The mature Notch receptor is then transported to the cell surface (Vinson et al. 2016).

Both Notch and its ligands are glycoproteins and their interaction is regulated by Notch glycosylation which alters the specificity of Notch for its ligands (Alberts et al. 2007). Determining events for selective activation of Notch by Jagged or Delta ligands are not only addition of sugar moieties on Notch by pofut (O-fucose) and poglut (O-glucose), but also addition of N-acetylglucosamine (GlcNAc) to the EGF repeats of Notch by the Fringe family enzymes of glycosyltransferases (Bigas and Espinosa 2018).

The signaling mechanism is initiated when the extracellular domain of Notch interacts with a Notch ligand which is located on the cell membrane of a neighboring cell (Kovall 2008). Notch can also be activated in an endocytic compartment within cells (Sancho et al. 2015) in a ligand-independent fashion (Palmer and Deng 2015). Mammals contain the Notch ligands Delta1, Delta3, Delta4, Jagged1 and Jagged2 while the Notch ligands in the model organisms *Drosophila melanogaster* and *Caenorhabditis elegans* are not the same as in mammals (Bertrand et al. 2012) (Figure 2).

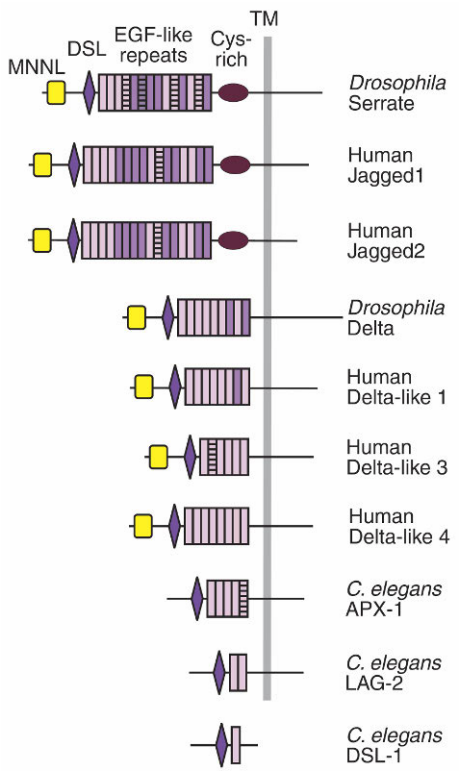

Figure 2. Schematic presentation of the structure of Notch ligands. The N-terminal region in all ligands, with the exception of that of *Caenorhabditis elegans,* contains a 100 amino acid long module at the N-terminus of Notch ligands (MNNL) region. All ligands comprise a cysteine rich module DSL close to/near the N-terminus which is followed by EGF-like repeats. Serrate, Jagged1 and Jagged2 also contain a cysteine rich domain. The figure is modified from (Gordon et al. 2008).

The dysregulation of Notch ligands is associated with developmental defects and cancer since the activation of the Notch signaling pathway depends so heavily on ligand binding (D'Souza et al. 2010). The Notch signaling pathway is an irreversible process and the receptor can be activated only once (Alberts et al. 2007).

A disintegrin and metalloproteinases (ADAMs) are zinc-dependent proteinases that are related to matrix metalloproteinases (Gibb et al. 2010). Notch is cleaved in its extracellular domain at site 2 (S2) by ADAM (Figure 3). When Notch is not activated by ligand binding, S2 is protected from cleavages by the receptor's negative regulatory region (NRR) (Bozkulak and Weinmaster 2009). Interaction with a Notch-specific ligand alters the conformation of Notch, including the NRR unfolding the structure of the NRR and thus exposing the ADAM cleavage site at S2 (Gordon et al. 2007; Palomero and Ferrando 2009). Ligand-activated Notch1 is prior to ADAM processing ubiquitinated by the E3 ubiquitin ligase DX4 (DTX4) resulting in the endocytosis of the Notch1 extracellular domain by the ligand expressing cell, whereas the membrane attached fragment of Notch and DTX4 are internalized by the Notch1 expressing cell (Chastagner et al. 2017). The structural change leads to extracellular proteolysis of Notch giving rise to the membrane tethered form of Notch, ΔEN (Gordon et al. 2007; Palomero and Ferrando 2009). Point mutations in the heterodimerization domain (HD) in the NRR weaken the stability of the NRR leading to ligand-independent cleavage of S2. These mutations occur in a large number of human T-cell acute lymphoblastic leukemia (T-ALL) cases (van Tetering et al. 2009). According to experiments performed by van Tetering et al., 2009, it is specifically the ADAM10 metalloproteinase Kuzbanian, but not ADAM17 that has the primary role in ligand induced extracellular cleavage at S2 (van Tetering et al. 2009).

The S2 cleaved Notch, ΔEN undergoes proteolytic cleavage which is catalyzed by the γ-secretase complex containing enzymatic activity. The cleavage occurs intracellularly at site 3 (S3) close to the plasma membrane, but the cleavage event can also occur in endosomes (Palaga et al. 2003). Cleavage at S3 generates NICD. In humans NICD1 has a mass of 97 kDa (De Strooper et al. 1999). Notch receptors that lack the extracellular domain are more

potently cleaved by the γ-secretase complex leading to the development of cancer, both in humans and mice (van Tetering et al. 2009). In humans, the complete form of Notch1 called full length Notch (FLN) consists of 2555 amino acids, ΔEN consists of the amino acids 1721-2555, and NICD the amino acids 1754-2555 (UniProtKB).

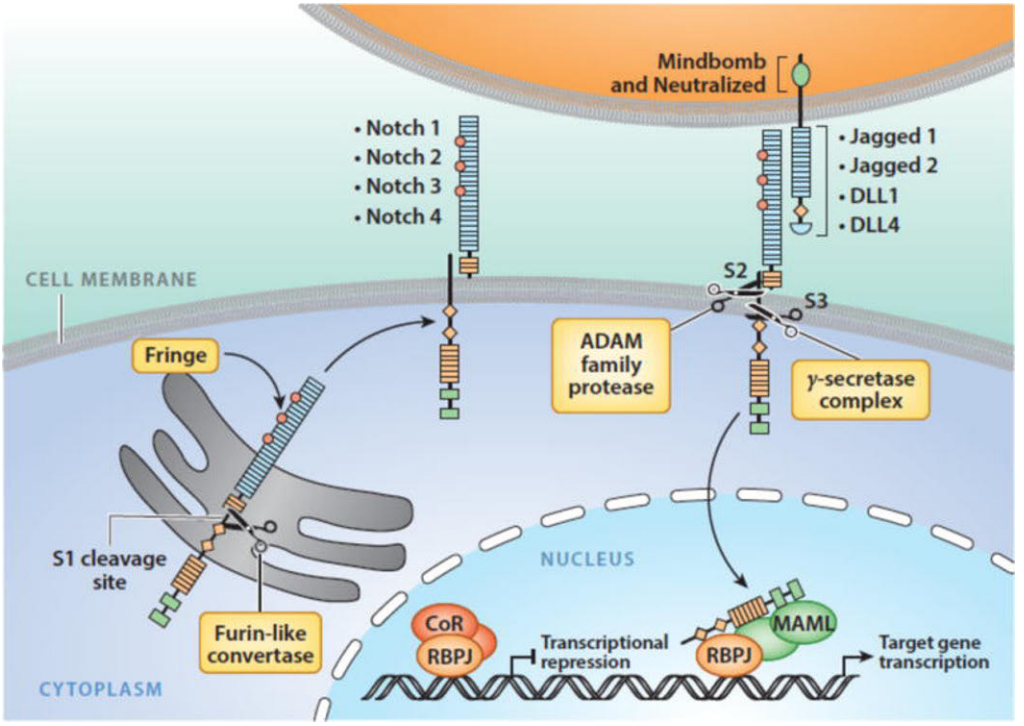

Figure 3. Activation of Notch by proteolytic cleavage. The first cleavage occurs in the Golgi complex at S1 after which the ready Notch heterodimer is presented in the plasma membrane. The ligand Delta or Jagged which is located on a neighboring cell, binds to Notch leading to the activation of the signaling mechanism. The complex consisting of the ligand and Notch subunit to which the ligand is bound is endocytosed to the ligand expressing cell. Thereafter Notch is cleaved extracellularly at S2 and quickly afterwards at S3. In canonical Notch signaling the cleaved Notch, now NICD, migrates to the nucleus where it binds to the transcriptional repressor CSL. This binding alters CSL to a transcriptional activator, which activates the transcription of Notch target genes. The figure is adapted from (Aster et al. 2017).

1.3 Transcriptional regulation of Notch target genes

Following proteolytic activation of ΔEN by the γ-secretase complex, NICD translocates to the nucleus where it binds to the transcription factor CSL (an acronym for CBF-1/RBPJ-κ in *Homo sapiens* and *Mus musculus*, *S*uppressor of Hairless in *Drosophila melanogaster*, *L*ag-1 in *Caenorhabditis elegans*) (Contreras-Cornejo et al. 2016). In mammals, CSL is also called Recombination signal binding protein for immunoglobulin kappa J region (RBPJ) (Osipo et al. 2008). CSL mediates the transcriptional activity of Notch and mediates signals from all four Notch receptor isoforms (Gao et al. 2009). The human CSL is sequentially 80% identical with the CSL from *Drosophila melanogaster* and 55% identical with the CSL from *Caenorhabditis elegans* (Kovall 2007).

1.3.1 Repressed state of Notch target genes

The default activity of CSL is considered to be repression of transcription (Dreval et al. 2019). In the absence of NICD, CSL functions as a transcriptional repressor by recruiting co-repressor proteins to form a transcriptional repressor complex (Borggrefe and Liefke 2012). This occurs by the local chromatin being converted to a transcriptionally silent form (Wilson and Kovall 2006). The highly evolutionarily conserved CSL whose DNA bound crystal structure was solved around 15 years ago (Kovall and Hendrickson 2004) binds to the motif CGTGGGAA (Tun et al. 1994).

In the absence of NICD, CSL maintains a repressive effect on Notch target genes in part by recruiting a histone deacetylase (HDAC) corepressor complex to the promoter of Notch target genes (Dreval et al. 2019). HDACs are divided into four classes based on function and DNA sequence similarity: class I (HDACs 1, 2, 3 and 8), class II (HDACs 4, 5, 6, 7, 9 and 10), sirtuin class III, and class IV (HDAC11) (Foti et al. 2013). It was discovered early on that in the absence of NICD, CSL interacts with a co-repressor complex that consists of nuclear receptor corepressor/silencing mediator for retinoid or thyroid-hormone receptors (NCoR/SMRT) and HDAC1 (Kao et al. 1998).

Early discovered co-repressors include also KyoT2 (Taniguchi et al. 1998) and CBF1 interacting corepressor (CIR) which binds to HDAC and SAP30 and thus functions as a linker between CSL and the HDAC complex (Hsieh et al. 1999). Other co-repressors include SMRT- and HDAC-associated repressor protein (SHARP, also known as MINT) (Oswald et al. 2002) which recruits additional co-repressors called CtIP/CtBP, ETO and NCoR/SMRT complex, the last mentioned linking CSL to HDACs (Giaimo et al. 2017). This means that a subset of corepressor complexes recruited by CSL contains histone deacetylases thereby generating a repressive chromatin landscape (Borggrefe and Oswald 2014).

Proteins of the co-repressor complex include also histone demethylases such as lysine-specific demethylase (LSD1, also known as KDMI1) which interacts with the NAD (+)-dependent deacetylase and gene repressor called silencing information regulator 1 (SIRT1). Together these two proteins have conserved roles in deacetylation of histone 4 lysine 16 (H4K16) and demethylation of histone 3 lysine 4 (H3K4) to repress Notch target genes (Mulligan et al. 2011). LSD1 demethylates H3K4 (Di Stefano et al. 2011) while class III

HDAC (Sirtuins) members SIRT1, SIRT2 and SIRT3 deacetylate H4K16 (Vaquero et al. 2007). SIRT1 does not form a complex only with LSD1, but with several histone modifying enzymes including HDAC1/2, EHMT1/2, and the repressor adaptor proteins CoREST1 and CtBP1, thus providing a role also for HDAC2 in a Notch target gene repressor complex (Mulligan et al. 2011). Relatively recently it was shown that in the absence of NICD, CSL also recruits L3MBTL3 (also known as MBT1) and the demethylase LSD1 to Notch target gene enhancers leading to H3K4 demethylation and consequently to transcriptional repression. L3MBTL3 competes with NICD for binding to CSL although the CSL-L3MBTL3 interaction is 20-fold weaker than the affinity of CSL-Notch RAM interaction (Xu et al. 2017). CSL has also been demonstrated to coordinate H3K27 deacetylation further enhancing repression of Notch target gene expression (Giaimo et al. 2017).

An integral part of the co-repressor complex is also the histone demethylase KDM5A (Liefke et al. 2010). Recently it was elucidated that HDAC1 negatively regulates the sequence-specific binding of CSL on mitotic chromatin in a KDM5A-dependent manner. The knockdown of HDAC1, or inactivation of HDAC1 with trichostatin A (TSA) leads to increased CSL presence on mitotic chromatin in a site-specific manner, and strikingly also to increased presence of KDM5A at these sites. Site-specific binding of KDM5A on mitotic chromatin is necessary for inceased CSL presence at these sites indicating that KDM5A positively regulates chromatin binding of CSL during these conditions. Once KDM5A binds to chromatin it can catalyze the demethylation of trimethylated H3K4 to the di- and monomethylated forms (Dreval et al. 2019).

1.3.2 Activation of Notch target genes

Binding of NICD to CSL displaces the co-repressors and activates CSL which recruits transcriptional Mastermind-like (MAML) and other coactivators to form a transcriptional activation complex bound to the DNA (Nam et al. 2003). The NICD-CSL interaction was shown first in *Drosophila melanogaster* (Fortini and Artavanis-Tsakonas 1994) and afterwards in mouse (Jarriault et al. 1995; Hsieh et al. 1996). CSL comprises three main domains: the DNA binding N-terminal and C-terminal domains, and the β-trefoil domain that binds to the RAM domain of NICD (Giaimo et al. 2017).

MAML1 interacts with the ankyrin repeat domain of all four mammalian Notch receptors and provides stability to the NICD-CSL interaction (Wu et al. 2000). MAML1 also recruits the histone acetyltransferase p300 which acetylates histones at the chromatin making the chromatin more open for transcriptional activation (Fryer et al. 2002). In addition, MAML1 potentiates p300 autoacetylation and thus increases its transcriptional activity (Hansson et al. 2009). The ternary complex consisting of NICD, CSL and MAML is a prerequisite for activating expression of Notch target genes in canonical Notch signaling (Kopan and Ilagan 2009; Wang et al. 2015). Loss of CSL leads to blockage of Notch target gene expression and therefore also to inhibition of Notch-driven physiological outcomes (Xu et al. 2017). Other members in the coactivator complex include the noncoding RNA steroid receptor coactivator (SRA), and the RNA helicase DDX5 (Jung et al. 2013) which for instance interacts with MAML1 in human T-ALL cells (Lin et al. 2013). CSL also interacts with the co-activating complex KMT2D/UTX which was relatively recently identified. KMT2D

methylates lysine 4 of histone 3 (H3K4), which in turn correlates with active chromatin (Giaimo et al. 2017).

The coactivator complex consisting of NICD, CSL and MAML and additional coactivators promotes acetylation of histones and activates Notch target genes (Aubin-Houzelstein et al. 2008; Borggrefe et al. 2016). On nucleosomes bordering the sites of the Notch transcriptional complex binding site, transcriptional activation has been shown to correlate with various PTMs of histones. In the promoters of Notch target genes, these PTMs include methylation of H3K4 and ubiquitination of histone H2B (Bray et al. 2005). In Notch-regulated enhancer elements these PTMs include acetylation of H3K27 (Wang et al. 2014) and H3K56 (Skalska et al. 2015). For the time being it seems plausible, based on mammalian cell research, that the major proportion of functional Notch regulatory elements are located specifically in enhancer elements, not in promoters (Aster et al. 2017).

The chromatin enhancer state affects which Notch target genes are responsive to activated Notch signaling. Recently the Brahma SWI/SNF chromatin remodeling complex including the actin-related BAP55 subunit was shown to have a conferring role in regulating the changes that affect how well CSL can bind to specific enhancers. The complex mediates increased nucleosome turnover at target enhancers leading to chromatin accessibility at these target enhancers for CSL to bind to. The histone turnover in nucleosomes is enhanced by Notch activity (Pillidge and Bray 2019).

In some cases, Notch target genes can be activated in a Notch-dependent, but CSL-independent way, i.e. without CSL interacting with Notch. This type of activation of Notch downstream target genes is generally referred to as non-canonical Notch signaling (Johnson and MacDonald 2011), but non-canonical Notch signaling includes additionally ligand-dependent, but γ-secretase-independent Notch signaling which has been shown to be involved in presynaptic protein expression in postmitotic neurons (Hayashi et al. 2016).

To add to the complexity of activation of Notch target genes, in addition to non-canonical Notch signaling, CSL can in some cases independently from Notch activate Notch target genes. Already in 1995 it was known that the Epstein-Barr virus EBNA2 is capable of turning the transcriptional repressive effect of CSL to transcriptional activation (Hsieh and Hayward 1995), but functions of Notch-independent functions of CSL have been difficult to reveal by genetic analyses since CSL-mediated and Notch-mediated functions often coincide and thus it has been difficult to know in which cases CSL-mediated functions have been affected by Notch. In addition, a particular Notch target gene may, depending on the case, be regulated by CSL independently or dependently of Notch. By combining data consisting of *cis*-regulatory sequences regulating detailed expression with gain and loss-of-function of CSL and Notch, elucidation of Notch-independent functions of CSL has emerged (Johnson and MacDonald 2011).

The DNA binding affinity of CSL is reflecting the fast pace of attachments and detachments of CSL to DNA. Proposed explanations to the mechanism that alters the CSL-DNA binding affinity have included the capability of co-factors to interpret the chromatin landscape, competition between CSL and other transcription factors for binding to the same enhancer site, and association of CSL and other DNA binding proteins prolonging CSL-chromatin interaction (Giaimo et al. 2017). The mechanism on how a repressive CSL is switched into

an activator at Notch target genes has been debated actively (Oswald and Kovall 2018). Most models have portrayed CSL to have long-lasting DNA-bound presence serving as a static platform protein that exchanges between co-repressors and NICD/coactivators. However, a study shows that in Notch-OFF conditions, a small proportion of CSL very transiently binds to DNA although this is essential for target loci repression, indicating that long-lasting DNA binding is not needed for repression. The same study highlights that in Notch-ON conditions, CSL presence increases drastically at its primary target locus E(spl)-C (Gomez-Lamarca et al. 2018).

1.3.3 The target genes Hes and Hey

The genes that the transcriptional complex can activate include members of for instance the gene family hairy/enhancer of split (Hes) consisting of Hes1-7 (Guo et al. 2011). The Hes target gene family encodes inhibitory gene regulating proteins and Hes is the primary target gene in Notch signaling in most cells even if the target genes vary depending on tissue type (Alberts et al. 2007). Hes has been shown to influence for example the development of endocrine cells, lymphocytes and pancreas (Fischer and Gessler 2007). More specifically, the Hes isoforms Hes1, Hes5 and Hes7 are known for their role as downstream Notch signaling effector proteins that regulate cell differentiation (Kobayashi and Kageyama 2014).

The gene family HES-related repressor protein (HERP), more often simply called Hey, is also one of the most central identified Notch target genes (Iso et al. 2003). The human Hey gene family comprises the genes of Hey1, Hey2, HeyL, HesL/HeIT, Dec1/BHLHB2 and Dec2/BHLHB3 (Guo et al. 2011). Expression of Hey1 does not always require Notch signaling. In such a case TGF-β induces fast and transient binding of endogenous Smad3/4 complexes at different Smad-binding element core repeats (SCRs) in the Hey1 promoter, occurring without Notch receptor activation or function. However, this immediate early activation cycle is followed by a second cycle of gene expression which requires traditional canonical Notch signaling (Zavadil et al. 2004).

Notch ligands have recently been demonstrated to activate different Notch target genes by activating the same Notch receptor isoform in either a pulsatile or a continuous manner. The Notch ligand Dll1 activates Notch1 in separate noncontinuous frequency-modified pulses that upregulate Hes1 expression whereas Dll4 activates Notch1 in a maintained amplitude-modified way which enhances Hey1 and HeyL expression. This indicates that the necessary information regarding ligand identity must be carried either within NICD dynamics or levels. Ectopic expression of the mentioned two ligands in chick neural crest also caused opposite outcomes in terms of myogenic differentiation (Figure 4) (Nandagopal et al. 2018), which is in line with an earlier study showing that Dll1 via Notch activation promotes muscle differentiation (Rios et al. 2011).

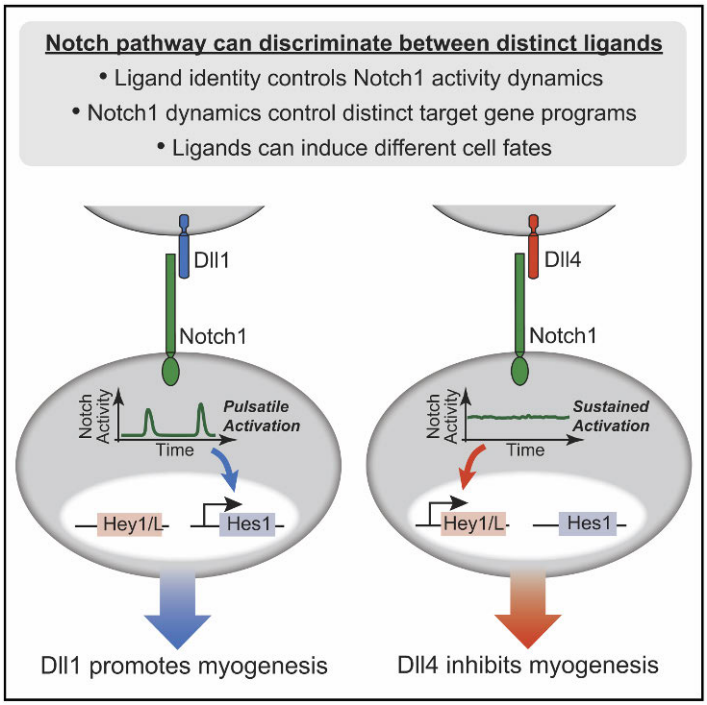

Figure 4. The Notch signaling pathway and its discrimination between ligands. The figure is adapted from (Nandagopal et al. 2018).

Dll4 has a higher binding affinity than Dll1 for Notch1 (Andrawes et al. 2013), which may partly explain the differences in activation manners of Notch by Dll1 and Dll4. Transcriptional activation of Hes genes as a cause of short pulse activation of Notch has been shown earlier (Housden et al. 2013), but this study lacked the elucidation of the involvement of Notch ligands, members of the Hey gene family, and outcomes of activated Hes/Hey gene expression. The context-dependent nature of Notch is reflected also in that Dll1 and Dll4 do not always mediate different differentiation effects. For instance, they both promote T-cell differentiation of hematopoietic progenitor cells (Mohtashami et al. 2010). For further details considering oscillatory pattern expression of Notch target genes, please see section "1.5.1 Notch in differentiation in the CNS".

Hes and Hey are the most extensively described Notch target genes, but after their identification as central Notch target genes, genome-wide screenings have revealed a larger set of Notch-regulated genes, including the cell morphogenesis genes Reck, talin and trio in adult muscle progenitor cells (Pezeron et al. 2014). Depending on circumstances and tissues, other Notch target genes include genes involved in the control of survival processes and the cell cycle, genes such as, Hif-1α, p27, nuclear factor kappa B (NF-κB) (transcriptional activator), Akt, vascular endothelial growth factor receptor (VEGF), Deltex (positive regulator of Notch activity), cyclin D1 (mitogenic sensor and allosteric activator of cyclin-dependent kinase CDK4/6), p21WAF1/Cip1 (cyclin-dependent kinase inhibitor functioning as an effector and sensor of several anti-proliferative signals), and Myc (oncogene and cell cycle regulator) (Guo et al. 2011; Lee et al. 2015). Specifically in tumors

with substantial gain-of-function Notch mutations, Myc is an often appearing target gene that seems to have an important role in augmenting Notch-dependent tumor cell growth (Aster et al. 2017).

Notch1 signaling regulates the activity of many factors that themselves are the most central regulators of other signaling pathways such as NF-κB, TGF-β, mTOR and PI-3K/Akt. Considering for example Akt, Hes1 binds to the promoter region of PTEN (a phosphatase and negative regulator of Akt signaling by dephosphorylation) and downregulates the PTEN expression thereby indirectly stabilizing Akt (Lee et al. 2015). In the beginning of the millennium it was reported that Hes1 potentially acts as a tumor suppressor in epithelial cells since induced Hes1 expression prevented the proliferative effect of 17beta-estradiol on breast cancer cells (Ström et al. 2000). The DNA binding specificity of the Hes and Hey proteins is determined by their basic domain, while the capability of these proteins to form homo- and heterodimers is determined by their helix-loop-helix domain (Guo et al. 2011).

1.3.4 Termination of Notch target gene expression

Hyperphosphorylation of NICD followed by degradation mediated by ubiquitin ligases terminates the Notch signal (Wilson and Kovall 2006). The exact events that shut down Notch signaling activity are not known, but it has been shown that the duration of Notch target gene transcriptions is partially regulated by the recruitment of cyclin-dependent kinase 8 (CDK8) to phosphorylate NICD (Vinson et al. 2016). The recruitment is performed by the transcription complex NICD-CSL-MAML and the ski-interacting protein SKIP which recruit kinases to the PEST and TAD domain of NICD. As a consequence of this, the phosphorylated PEST domain is recognized by the F-box/WD40 domain-containing protein 7 (FBW7/SEL10) E3 ubiquitin ligase which mediates NICD ubiquitination, leading to the proteosomal degradation of NICD (Figure 5) (Bray and Gomez-Lamarca 2018).

Point mutations in conserved serine residues within the NICD PEST domain prevents hyperphosphorylation by CDK8 and instead stabilizes NICD (Fryer et al. 2004). Proteosomal degradation of NICD is hindered also by FBW7 mutations making FBW7 unable to recognize the phosphorylated NICD region (O'Neil et al. 2007). For further details considering ubiquitin-mediated degradation of NICD, please see section "1.4.2 Ubiquitination" in section "1.4 Post-translational modifications of Notch". Following NICD degradation, the transcriptional repressors reassemble a repressing complex preventing the activation of Notch target gene transcription (Lee et al. 2015).

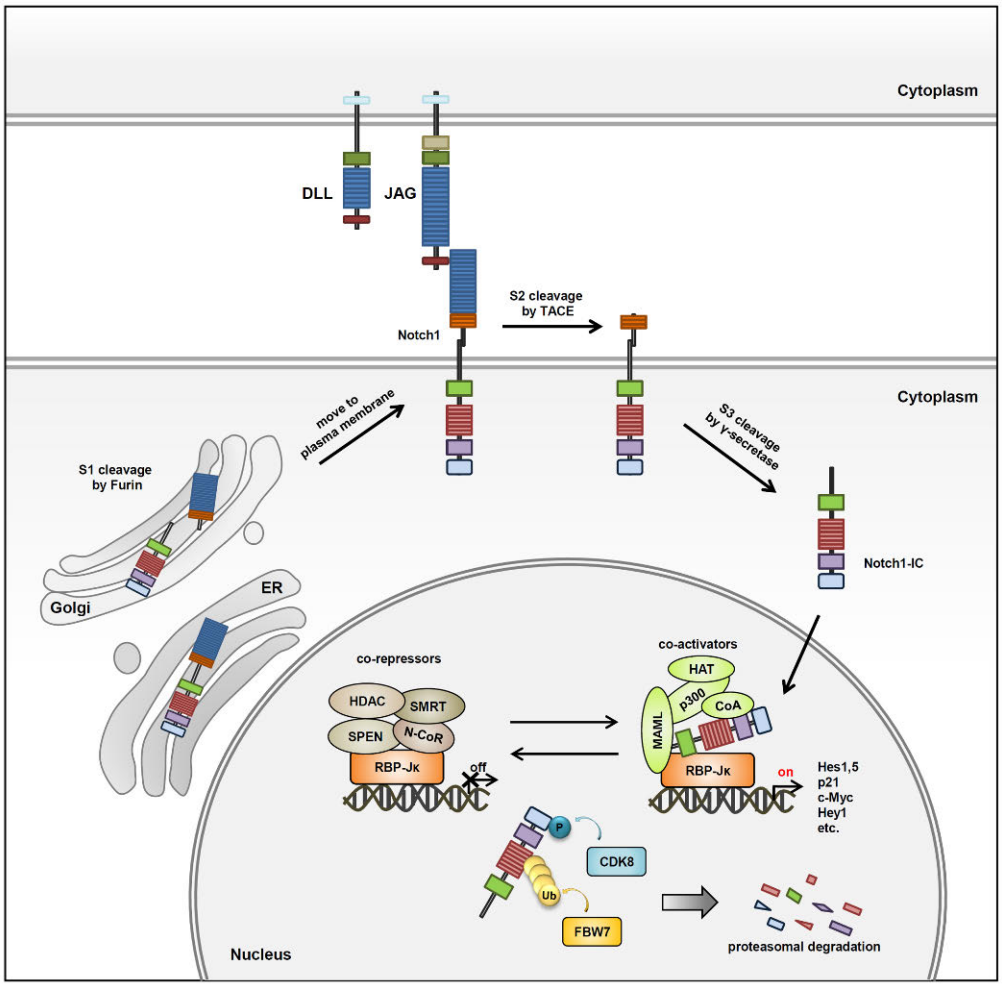

Figure 5. The Notch signaling pathway and its nuclear cycle. NICD migration into the nucleus and binding to CSL/RBP-Jκ displaces the repressive complex, and activates of Notch target genes. Termination of the transcription is mediated by CDK8 and FBW7/SEL10. HDAC: histone deacetylase, SMRT: silencing mediator for retinoid and thyroid hormone receptors, SPEN: split-ends, N-CoR: nuclear receptor corepressor, MAML: Mastermind, HAT: histone acetyltransferase, CoA: coactivator. The figure is adapted from (Lee et al. 2015).

Other mechanisms to downregulate Notch target gene expression also exist. RBP-J interacting and tubulin associated (RITA) interferes with the transcription mediated by Notch and CSL. RITA shuttles between the nucleus and the cytoplasm and mediates the nuclear export of CSL to tubulin fibers. This way RITA functions as a transcriptional repressor of an already activated Notch signaling (Wacker et al. 2011).

1.4 Post-translational modifications of Notch

Cells experience constant regulation of transcription and translation. Which, how many and how often proteins are being synthesized are defined by the internal and external requirements of the cells. Synthesized proteins may thereafter be modified post-translationally, which can regulate the localization, interactions, function and stability of the target protein. A post-translational modification (PTM) indicates addition of a chemical group or a small protein to the target resulting in its modification.

Regulation of the activity of Notch signaling can be mediated by crosstalk with other signaling pathways and by PTMs (Andersson and Lendahl 2014). Notch signaling is regulated by PTMs that influence nuclear translocation, target gene expression, and half-life of NICD (Andersson et al. 2011; Lee et al. 2015). Notch is a target of several post-translational modifications that have a strong impact on Notch transcriptional activity. These PTMs include for instance sumoylation, phosphorylation and methylation (Figure 6.)

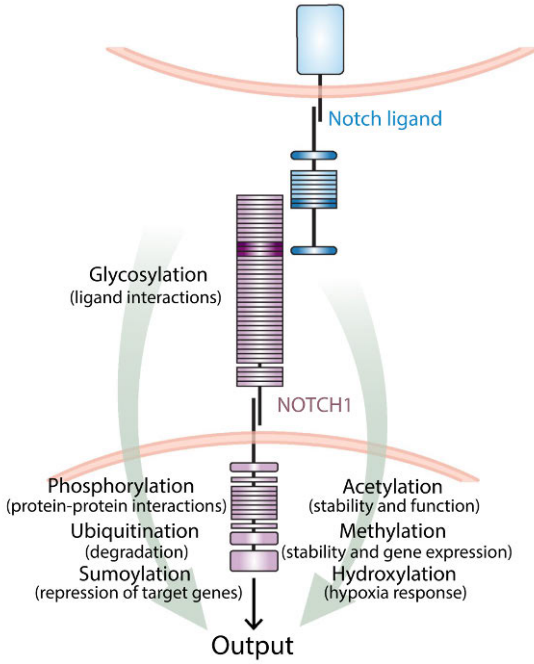

Figure 6. Notch1 and its ligands are targets for several PTMs that regulate different aspects of the signal transduction. Some of the PTMs essentially affect Notch target gene expression and consequently biological outcomes such as tissue differentiation as in the case of NICD1 methylation. The figure is adapted from (Antfolk et al. 2019).

1.4.1 Sumoylation

The post-translational modification mediated by small-ubiquitin like modifier (SUMO) proteins regulates a wide variety of cellular functions including transcription, macromolecular assembly, chromatin organization, protein homeostasis, signal transduction, trafficking and DNA repair (Flotho and Melchior 2013). Sumoylation has emerged as an essential regulator of various aspects of nuclear functions including transcriptional regulation (Henley et al. 2014). The roles of sumoylation within the Notch pathway, however, have only started to be uncovered in the last few years. It has previously been indicated that sumoylation inhibits one or more components in the LIN-12/Notch

signaling pathway in *Caenorhabditis elegans* (Poulin et al. 2005). More evidence of SUMO modification regulating Notch signaling was obtained when it was shown that the LIM domain protein KyoT2 is a target protein for SUMO2/3 in a protein inhibitor of activated STAT1 (PIAS1)-catalyzed reaction. It was also shown that sumoylation enhances the transcriptional activity of KyoT2 on the Notch signaling pathway. KyoT2 is known to be recruited by RBP-J/CSL to function as a transcriptional repressor for Notch target genes (Wang et al. 2007b). By inhibiting global sumoylation by prevention of the E1-activating complex SAE1/UBA2 and knockdown of the E2-conjugating enzyme Ubc9, the growth of Notch1-activated breast epithelial cells is decreased (Licciardello et al. 2015). Cellular stress induces conjugation of SUMO1 and SUMO2/3 to NICD1 in the nucleus and represses the expression of the classical Notch1 target genes Hes1, Hey1 and Hey2 (Antila et al. 2018). It has also been shown that NICD1 sumoylation is increased in mesenchymal stem cells (MSCs) with stabilized hypoxia-inducible factor 1 alpha (HIF-1α) expression, leading to cell migration and invasiveness. With stabilized HIF-1α expression, increased NICD1 sumoylation was also detected in HEK293T cells (Ciria et al. 2017). Interestingly, a SUMO proteomics strategy has recently indicated Notch2 to undergo conjugation by SUMO at K1353 and Notch3 at K2061 (Hendriks et al. 2017). The physiological functions of these sumoylations are still to be discovered.

Notch1 sumoylation has a role in the endothelium. According to relatively recent data, by targeting endothelial Notch signaling, sumoylation negatively regulates angiogenesis. Deletion of endothelial sentrin-specific protease 1 (SENP1) which is the primary protease in sumoylation, enhances endothelial Notch sumoylation and prolongs Notch1 signaling, which suppresses vascular endothelial growth factor (VEGF) receptor signaling and angiogenesis (Zhu et al. 2017). PIAS1 catalyzed sumoylation of Hes1 induced by oxidative stress represses the expression of GADD45α, which increases cell survival (Chiou et al. 2014). Recently it has been shown that overexpression of the desumoylating protein, sentrin-specific protease 2 (SENP2) suppresses Notch signaling whereas silencing of SENP2 leads to upregulation of the Notch signaling activity (Chen et al. 2018).

MAML1 in the NICD1 transcriptional complex is a subject for SUMO conjugation, which enhances the MAML1-HDAC7 interaction causing decreased MAML1 transcriptional activity (Lindberg et al. 2010). Crosstalk between Notch sumoylation and other PTMs of Notch may also occur. For instance, in a screen that identified over 2,500 proteins being targets for SUMO E3 ligase-dependent sumoylation, revealed that kinases were particularly often conjugated by SUMO, indicating crosstalk between phosphorylation and sumoylation (Uzoma et al. 2018). In addition, a site-specific mapping identified over 800 sumoylated peptides that were co-modified with phosphorylation (Hendriks et al. 2017). In another recent screen which focused on the crosstalk between sumoylation and ubiquitination, the authors described an improved method for enrichment of co-modified proteins. The screen identified 498 proteins which are modified by both sumoylation and ubiquitination (Cuijpers et al. 2017).

1.4.2 Ubiquitination

Ubiquitination adds ubiquitin groups to lysine residues modifying cellular localization and protein stability, with polyubiquitination being the standard tag for degradation by proteasomes (Moretti et al. 2012). Regarding post-translational modifications of Notch, ubiquitination is so far the best described and characterized (Hein et al. 2015). The lifespan of Notch is fairly short and its degradation occurs predominantly through polyubiquitin conjugation (Bielskienė et al. 2015).

Many different ubiquitin E3 ligases have been discovered to ubiquitinate NICD. Ubiquitin conjugation of the membrane tethered Notch1 by Itch (Qiu et al. 2000) is facilitated by the protein Numb, which also promotes the degradation of NICD1 (McGill and McGlade 2003). Recently it was shown that Shootin1 enhances the activity of the Notch pathway by interacting with LNX1/2 and stimulating Numb ubiquitination, or by forming a complex with Itch and inhibiting NICD ubiquitination (Sapir et al. 2017). In *Caenorhabditis elegans*, the E3 ubiquitin ligase Ubiquitin protein ligase E3 component n-recognin 5 (UBR5) negatively regulates GLP-1 and LIN12 activity, the two Notch receptors expressed by *Caenorhabditis elegans* (Safdar et al. 2016).

Although ubiquitination of Notch does not always cause repressed Notch levels, as discussed in this section, ubiquitination-mediated proteasomal degradation of Notch is crucial to fine-tune the half-life of NICD. Failure in preventing prolonged Notch signaling causes serious diseases, such as different forms of cancer including T-cell acute lymphoblastic leukemia (T-ALL) (Demarest et al. 2008). Ubiquitination of Notch also crosstalks with other PTMs of Notch. It has been shown that the C-terminal PEST domain of NICD1 is phosphorylated leading to ubiquitination of NICD1 by FBW7/SEL-10, which negatively regulates Notch signaling by targeting Notch for proteasome-mediated degradation (Hubbard et al. 1997; Oberg et al. 2001; Wu et al. 2001). Flaws in NICD-FBW7/SEL10 interaction are associated with leukemia and many solid cancers (Carrieri and Dale 2017). For instance, in adult T-cell leukemia (ATL) FBXW7/SEL10 mutant proteins are defective to interact with NICD resulting in increased NICD levels and prolonged Notch signaling (Yeh et al. 2016). Interestingly, FBW7/SEL10 mutations in leukemic cells do not only enhance the signaling efficiency of Notch, but also mediate resistance to γ-secretase inhibitors (O'Neil et al. 2007). Recently it was shown that also the E3 ubiquitin ligase ring finger protein 8 (RNF8) ubiquitinates NICD1 resulting classically in the degradation of NICD1. In addition, low expression of full-length RNF8 correlates with bad prognosis for breast cancer patients (Li et al. 2018).

However, ubiquitination does not always result in decreased NICD levels. Ubiquitination of NICD by the RING ubiquitin ligase RNF4 stabilizes NICD and enhances the transcriptional activity of NICD (Thomas et al. 2016). NICD transcriptional activity is also stimulated as a consequence of NICD ubiquitination by murine double minute 2 (MDM2) (Pettersson et al. 2013). MDM2 also ubiquitinates NICD4, which stimulates an NICD-Trp53 interaction, which in turn represses NICD4-induced anchorage-independent growth in mammary epithelial cells (Sun et al. 2011). In addition, monoubiquitination of Notch is a prerequisite for γ-secretase cleaving ΔEN (Gupta-Rossi et al. 2004).

Ubiquitination of Notch is balanced to some extent by deubiquitinases which generally slow down the ubiquitin-dependent turnover of Notch. The utilization of an *in vivo* RNA interference (RNAi) screen led to the discovery of several DUBs that regulate Notch signaling (Zhang et al. 2012). The deubiquitinating protein ubiquitin-specific peptidase 10 (USP10) interacts with NICD1 counterbalancing the ubiquitin-mediated degradation of Notch. In the endothelium, the Notch-USP10 decreases angiogenic sprouting providing a fine-tuning regulatory role for USP10 through Notch (Lim et al. 2019). The loss of the deubiquitinating protein Ubiquitin carboxyl-terminal hydrolase 5 (USP5) leads to the upregulation of Notch during *Drosophila melanogaster* eye development (Ling et al. 2017). The HES1 protein is a target for the E3 ubiquitin ligase SCF^{FBXL14} complex resulting in HES1 proteolysis, which further enhances neuronal differentiation (Chen et al. 2017). The plot thickened recently regarding the balance between Notch ubiquitination and deubiquitination when it was demonstrated that the protein Numb also facilitates the association between NICD1 and the deubiquitinase BAP1 which in turn stabilizes NICD1, yet independently of its deubiquitinase activity. By enhancing Notch signaling, BAP1 maintains stem-like properties of neural progenitor cells (Luo et al. 2019).

Also Notch ligands may be subjects of ubiquitination, reviewed in [(Weinmaster and Fischer 2011; Liu et al. 2016)]. The ubiquitin E3 ligase Neuralized (Neur) interacts with the Notch ligand Delta1 and represses its levels in *Xenopus laevis* (Deblandre et al. 2001) and *Drosophila melanogaster* (Lai et al. 2001). For Notch signaling to be efficiently activated by Delta, the intracellular domain of the latter protein needs to interact with the ubiquitin ligase Mind Bomb. The interaction promotes the ubiquitination and internalization of the ligand (Itoh et al. 2003).

Also, other proteins within the transcriptional activator protein complex NICD-CSL-MAML have shown to be targets of ubiquitination. For instance, ubiquitin conjugation to MAML1 is inhibited by NICD1, but enhanced by p300, and decreased MAML1 ubiquitination causes increased transcriptional output in a Hes1 reporter assay (Farshbaf et al. 2015).

1.4.3 Glycosylation

Glycosylation adds mono- or polysaccharides (glycans) to a recipient molecule which can be either a saccharide, lipid or protein. Both Notch and its ligands undergo glycosylation in the form of addition of sugar moieties, which causes structural changes in the modified proteins fine-tuning the affinity of Notch for its ligands. Notch is subject to glycosylation during migration through the ER and Golgi network where glycosyltransferases add glycans to Notch. Notch extracellular domain (NECD) is glycosylated in its 29-36 EGF-like repeats to develop as a mature Notch receptor and become localized on the cell surface. The NECD is glycosylated by a variety of different carbohydrates including asparagine linked N-glycans and many distinct threonine or serine linked O-glycans. The types of monosaccharides that are bound to threonine or serine amino acids of proteins determine how the O-glycans are classified. These EGF-repeats are subject to modification by O-glucose, O-fucose, O-GlcNAc and O-xylose. Fucosylation is the dominating form of glycosylation in Notch. On the contrary to the glucose residues, xylosylation decreases Notch signaling activity (Takeuchi and Haltiwanger 2014; Pakkiriswami et al. 2016).

1.4.4 Acetylation

The transfer of acetyl groups to lysine residues from acetyl coenzyme A is termed acetylation. Several acetyltransferases have been discovered to add acetyl groups to Notch. Notch1 is a target of acetylation by the histone acetyltransferase Tip60 leading to suppression of Notch signaling activity (Kim et al. 2007). Other acetyltransferases acetylating Notch1 include PCAF and GCN5 (Kurooka and Honjo 2000) and the transcriptional coactivator p300 (Oswald et al. 2001). p300 acetylates not only Notch1, but also MAML1 whose acetylation by p300 is a prerequisite for initiation of Notch-dependent transcription (Jin et al. 2017). Acetylation of NICD1 by p300, which is mediated by MAML1 and CSL, lessens the ubiquitination and the subsequent degradation of NICD1 and thereby stabilizes NICD1 (Guarani et al. 2011; Popko-Scibor et al. 2011). Notch1 undergoes deacetylation by SIRT1 (Guarani et al. 2011), and Notch3 by histone deacetylase 1 (HDAC1) stabilizing Notch3. In contrast to NICD1, acetylation of NICD3 by p300 enhances NICD3 ubiquitination and consequent degradation (Palermo et al. 2012).

1.4.5 Phosphorylation

Phosphorylation is defined as addition of phosphate groups from ATP to serines, threonines or tyrosines. NICD1 phosphorylation crosstalks with NICD1 ubiquitination. Phosphorylation of NICD1 in its C-terminal PEST domain results in the ubiquitination of NICD1 and consequent degradation of NICD1 providing an indirect role for phosphorylation in the regulation of NICD1 stability (Hubbard et al. 1997; Oberg et al. 2001; Wu et al. 2001). A wellknown phosphorylation of NICD1 is performed by cyclin C/cyclin-dependent kinase 8 (CDK8) which phosphorylates NICD1 in its PEST domain targeting NICD1 for ubiquitination by FBW7/SEL10 ubiquitin ligase and subsequent proteasomal degradation (Fryer et al. 2004). But phosphorylation may in some cases also enhance NICD1 stability. For example, it has been demonstrated that NICD1 is subject to phosphorylation by glycogen synthase 3 β, leading to stabilization of NICD1 (Foltz et al. 2002; Espinosa et al. 2003). PIM kinases phosphorylate NICD1 and regulate NICD1 nuclear localization and transcriptional activity (Santio et al., 2016). Furthermore, Notch is phosphorylated by many different kinases affecting both Notch stability and function. These have been reviewed for instance in [(Lee et al. 2015; Borggrefe et al. 2016)].

1.4.6 Hydroxylation

Hydroxylation is defined as the addition of a hydroxyl group to a side chain of an amino acid. The asparaginyl hydroxylase factor inhibiting HIF (FIH) catalyzes the hydroxylation of two strongly conserved asparaginyl residues that are located in the ankyrin repeat domains of NICD1-3 (Wilkins et al. 2009). Hydroxylation of NICD1 by FIH is critical for NICD1 functioning properly both during neurogenesis and myogenesis. FIH dampens Notch signaling activity and enhances myogenic differentiation (Zheng et al. 2008). Hydroxylation of NICD does not apparently affect the interaction of NICD to CSL, Ski-interacting protein (SKIP) or Deltex (Borggrefe et al. 2016). The cells' response to emerged hypoxia is to stabilize HIFα that will directly activate transcription of its downstream target genes (Zheng et al. 2008). Hydroxylation of Notch by FIH also indirectly regulates oxygen sensing by competing with the hydroxylation of HIFα by FIH. NICD1 hydroxylation by

FIH thus decreases the hydroxylation of HIFα and consequently increases HIF signaling. The binding affinity of FIH is greater for Notch than for HIFα (Wilkins et al. 2009). During hypoxia the hydroxylation of NICD1 by FIH is greatly minimised (Coleman et al. 2007).

1.4.7 Methylation

Methylation adds a methyl group to a lysine or arginine amino acid within a protein. In the nucleus, NICD1 is methylated at five conserved arginine residues in the C-terminal transactivation domain by coactivator-associated arginine methyltransferase 1 (CARM1), which enhances NICD1 transcriptional activity. CARM1 which is a protein arginine methyltransferase (PMRT) interacts not only with NICD1, but also with CSL in the NICD-coactivator complex, and is also present at Notch gene enhancers in a Notch-dependent way. A methylation-defective NICD1 mutant is more stable than a WT NICD1, which would indicate that methylation of NICD1 by CARM1 increases the proteasome-mediated degradation of NICD1 despite that methylation of NICD1 by CARM1 increases the transcriptional activity of NICD1 (Hein et al. 2015). In addition to Notch1 undergoing methylation, also Notch3 has been indicated to be a target of arginine methylation in the human colon cancer cell line HCT116 (Guo et al. 2014).

1.5 Notch in cell differentiation and tissue specificity

The regulation of cell fate decisions is critical in the formation of fully functional organs and maintenance of healthy tissue homeostasis. Notch signaling is utilized in many different developmental settings both in embryonic and adult tissues (Sancho et al. 2015). The functional consequences of Notch signaling are dependent on the cellular context as Notch signaling can either **induce or block stem cell differentiation**, stimulate survival, proliferation or cell death (Hori et al. 2013). In most cases the central role of Notch in biological functions is associated with cell differentiation (Bellavia et al. 2007). Organisms use different signaling mechanisms to differentiate precursor cells to specialized tissues and organs. Notch signaling is critical among these signaling pathways (Kovall 2007).

The biological outcomes of Notch signaling can vary also depending on the interplay between Notch signaling and other transduction pathways such as Wnt, Hedgehog, JAK/STAT and TGFβ/BMP, which can either increase or decrease Notch activity (Nemir and Pedrazzini 2008). Also, the signaling mechanism NF-κB crosstalks with Notch whose activity causes increased NF-κB signaling. In turn, an increased activity of NF-κB stimulates Notch activity by amplifying the expression of Notch receptors and ligands (Poellinger and Lendahl 2008). The N-terminal region of Notch1 crosstalks with p53, which inhibits the phosphorylation of p53 and diminishes its transactivation capacity (Kim et al. 2006).

One of the most studied aspects of Notch signaling in mammalian cells is how Notch affects different stem cell systems during embryonic and post-embryonic development (Chiba 2006). Notch1 and Notch2 are expressed broadly in many tissues during development and adulthood. Mice lacking either the Notch1 or the Notch2 gene die during embryogenesis as a consequence of developmental defects in several organs. However, Notch3 and Notch4

knockout mice are viable since they have phenotypes that are mostly constrained only to blood vessels. Notch3 is expressed mainly in vascular smooth muscle and pericytes, and Notch4 is expressed mostly in the endothelium (Aster et al. 2017).

By regulating cell fate during embryonic development, Notch affects the establishment of tissue specificity of stem cells (Tsivitse 2010). Notch regulates stem cells in several different settings such as the nervous system, skin and the development of blood cells, and the colon (Mizutani et al. 2007). Notch is also involved in tissue homeostasis in adults by maintaining and repairing tissues (Luo D1, Renault VM 2005). Signaling via Notch1 and Notch2 is a prerequisite for maintaining the population of melanocyte stem cells in mice (Schouwey et al. 2010).

Analyses of both upregulated and downregulated Notch signaling have indicated an effect on the development of T-cells and B-cells. Deletion of the genes that encode for Notch1 or CSL in the bone marrow of adult mice leads to deterioration of early T-cell development and disturbances in the differentiation of immature B-cells in the thymus (Tanigaki and Honjo 2007). In the absence of Notch1, bone marrow precursor cells migrate to the thymus and differentiate into B-cells, which suggests that Notch1 mediates functions that cause lymphatic stem cells to develop into T-cells instead of B-cells (Radtke et al. 2004).

1.5.1 Notch in differentiation in the CNS

In vertebrates, the nervous system consists of the peripheral nervous system (PNS) and the central nervous system (CNS), the latter one consisting of the brain and spinal cord. The brain consists of about 100 billion neurons that send and receive electro-chemical signals to and from the nervous system. In addition, the brain and spinal cord are made up of glial cells that build up myelin, regulate homeostasis, and also provide protection and support for neuronal cells. The CNS is important for neuroendocrine function and regulates glucose and energy metabolism (Bi and Kuang 2015). Throughout the entire brain, neural cells are being matured from earlier progenitor cells that line the ventricular zone (Biehl and Raetzman 2015). In the subventricular zone, neural stem cells that proliferate, most likely do so for self-renewal purposes and formation of intermediate progenitors (Giachino and Taylor 2014). Neural stem cells refer to all CNS stem cells whereas neuronal stem cells refer to CNS progenitor cells dedicated to differentiating into neurons.

The Notch signaling mechanism regulates the timing of neural progenitor cell differentiation in metazoans (Ratié et al. 2014). Already in the 1990's it was known that Notch by inducing the endogenous expression of the Notch downstream target genes Hes1 and Hes5 inhibits neural differentiation (Ohtsuka et al. 1999) and thus maintains neural progenitor cells as neural progenitor cells (Ratié et al. 2013). The knowledge of the role of Notch as a critical regulator of cell differentiation in the CNS has significantly increased in this millennium. The Notch transduction mechanism regulates the proliferative ability of cultured neural stem cells, and the Notch downstream target gene product Hes1 controls the amount and ratio of neurons and glia being produced from earlier stage cells (Aujla et al. 2013).

The Hes1 and Hes5 proteins are critical in maintaining an appropriate amount of progenitor cells during development of the neocortex. Depletion of the mentioned two proteins leads to an inappropriate loss of progenitor cells by enhancing premature differentiation of progenitors to neurons. Accordingly, prolonged activation of Notch1 maintains progenitor cells in their current cell state and prevents their differentiation into neuronal cells (Biehl and Raetzman 2015). The defectively low activities of the Hes1 and Hes5 proteins result in neural stem cells not being able to sustain an appropriate undifferentiated cell state, but will lead to cells differentiating when they should not. This again will cause several defects in brain structure and cell morphology. Notch1 knockout results in neural stem cells not being able to proliferate when they should and instead they end up in an inappropriate and premature differentiation (Zhang et al. 2018a). In addition, it has been shown that Hes1 and Hes3 double null mice develop too early neural differentiation in the mesencephalon (midbrain) and rhombencephalon (hindbrain) (Ratié et al. 2013). Adding to the regulation of Notch-mediated impact on neurogenesis, Hes6 has been decribed as an inhibitor of Hes1 during neuronal development (Hartman et al. 2009).

In the nervous system, Notch controls a regulatory loop between neighboring cells termed lateral inhibition. In this case, when a stem cell differentiates into a neuron in a group of stem cells, the differentiating stem cell starts to express Notch ligands. The ligands activate Notch signaling in its neighboring cells where the Notch target genes encode proteins that inhibit genes that now are prevented from encoding the Notch ligands Delta-like and Jagged. Therefore, these neighboring cells do not undergo neural differentiation, but develop into epidermal cells instead (Zhu et al. 2006).

More specifically, as with intestinal epithelial progenitor cells (described in "1.5.2 Notch in differentiation in the intestine"), when a progenitor cell starts to differentiate, the expression of the Notch ligand Delta gene is upregulated, which activates the Notch cascade in its neighboring cell where ultimately gene repressor proteins Hes/Hey are synthesized. These proteins block the expression of genes required for differentiation such as **Ascl1** (formerly known as Mash1) and **Neurogenin1/2** (Ngn1/2) belonging to the NeuroD gene family, resulting in the maintenance of neural progenitor cells in their progenitor cell state (Ratié et al. 2013). The members of the proneural gene family **NeuroD are in addition to activating neural differentiation also capable of activating expression of Notch ligand genes** including Dll1 (Figure 8) (Zhang et al. 2018b). Thereby, blocking Ascl1 and Ngn1/2 expression also causes repression of Delta expression (Figure 7) (Ratié et al. 2013).

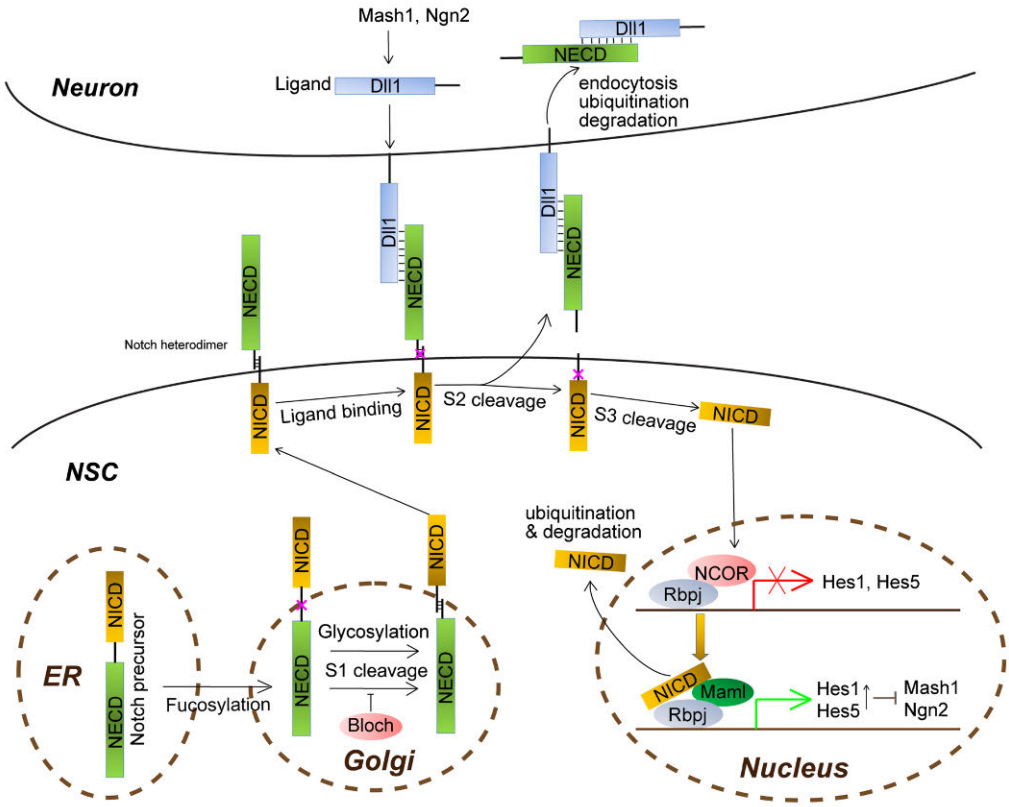

Figure 7. The Notch signaling pathway and its regulation in the CNS. The proneural genes and transcriptional activator proteins Ascl1 (formerly known as Mash1) and NeuroG1/2 (also known as Ngn2) initiate the expression of the Notch1 ligand Dll1 in differentiating cells. The expressed Dll1 in turn will activate Notch signaling in its neighboring cells where Hes1 and Hes5 proteins will be synthesized blocking the expression of the proneural genes Ascl1 and NeuroG1/2 and will thus prevent neuronal differentiation. The figure is adapted from (Zhang et al. 2018b).

Hes-mediated oscillation caused by hypostable Hes-mRNA and proteins is essential for neurogenesis to take place appropriately from a regulatory standpoint of view. Here oscillation refers to regulatory repetitive variation in time (Zhang et al. 2018b). By time-lapse imaging it has been demonstrated that the Hes1 gene, the proneural genes Ascl1 and Ngn2, and the Notch ligand Dll1 gene are dynamic (adaptive/changing) in neural progenitor cells and are expressed in an oscillatory/cyclic mode (Hirata 2002; Masamizu et al. 2006; Shimojo et al. 2008; Imayoshi et al. 2013), indicating that Hes1 oscillation regulates Ascl1, Ngn2 and Dll1 oscillations (Shimojo et al. 2008). However, Ascl1 is not always expressed in an oscillatory manner. Although sustained Ascl1 expression enhances neuronal differentiation, cyclic/oscillatory expression of Ascl1 maintains proliferating neural progenitors (Imayoshi et al. 2013). The oscillation of Hes gene expression regulation is possibly critically important in the outcome of whether a neural stem cell is maintained in its current progenitor cell state or whether its differentiation is induced. Hes proteins

actually compete with the NICD-CSL-MAML transcriptional activator complex at the Hes1 and Hes5 promoters to negatively regulate their own expression. Notch and its ligands are widely expressed throughout neurogenesis (Figure 8) (Zhang et al. 2018b).

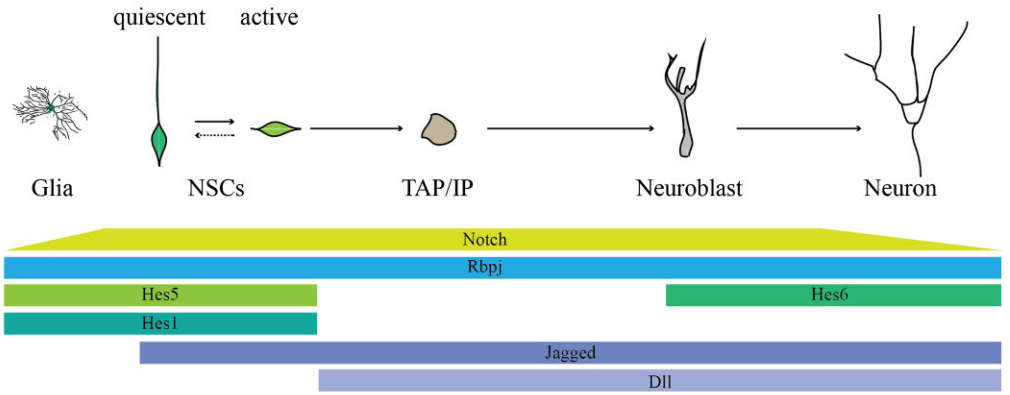

Figure 8. Notch signaling components in neural development. The Notch receptor, and the Notch target gene transcriptional activator CSL (referred to here as Rbpj) gene are expressed throughout neurodevelopment, whereas the Hes genes, and the Notch ligand genes Jagged and Dll1 are expressed in a smaller amount in different cell types as indicated. The figure is adapted from (Zhang et al. 2018b).

Therefore, high Notch activity holds neural stem cells in a proliferative state preventing differentiation whereas low Notch activity causes neural stem cells to stop proliferating and instead initiate differentiation (Zhang et al. 2018a). The consequences considering differentiation in the CNS are similar if CSL is knocked out instead of Notch1. In the brain of tamoxifen-inducible conditional CSL knockout mice, complete absence of neuronal stem cells takes place as depletion of CSL results in overactive and transient differentiation of neuronal stem cells into neurons. It has been shown that cerebral vessel endothelial cells enhance the quiescence of neural stem cells by having Jagged1 activate Notch signaling in its neighboring cell. In accordance, removal of Jagged1 facilitates abnormal activation of quiescent neural stem cells towards differentiation (Bi and Kuang 2015).

However, Notch does not only regulate whether cells should or should not proliferate or differentiate and when or when not to do so. Cells that have already begun their differentiation are strongly affected by Notch signaling in regards to which cell fate state these cells eventually differentiate (Biehl and Raetzman 2015). The role of Notch determining final cell fate state of for instance intestinal epithelial transit amplifying (TA) progenitor cells is described in the following section.

1.5.2 Notch in differentiation in the intestine

For more general concepts considering the anatomy of the colon, and proliferation and differentiation of colonic epithelial stem cells and progenitor cells, please see section "3.1.1 Proliferation and differentiation of colonic epithelial stem cells and progenitor cells".

Notch and the Wnt signaling pathways are considered critical for the regenerative ability of the intestinal epithelium (Naito et al. 2017). The Notch signaling pathway has an essential role in regulating the stem cell fate within the intestinal epithelium. In the developing and adult intestine both the Notch receptors and its ligands are expressed in the intestinal epithelial tissue, mostly in the invaginations called crypts (Roulis and Flavell 2016). Within the crypts, the largest amounts of Notch and Wnt are found at the bottom of the crypts (Vinson et al. 2016). In addition to Notch and Wnt signaling, major players controlling stem cell fate in the intestinal crypts include bone morphogenic protein (BMP) signaling pathway and Hedgehog (Park et al. 2018).

The Notch signaling mechanism activated by the Notch ligands Dll1 and Dll4 is highly needed for the accurate regulation of the intestinal stem cell niche where it hinders the differentiation of stem cells and instead promotes proliferation and survival of stem cells which are located at the bottom of intestinal crypts (Pellegrinet et al. 2011). The contribution of Notch to the maintenance of the proliferative crypt occurs by Notch-mediated Hes1 expression leading to the transcriptional repression of the CDK inhibitors **p27Kip1** and **p57Kip2** (Riccio et al. 2008).

The Notch cascade also regulates the proliferation of transit TA cells, and enhances differentiation of TA cells to the absorptive cell lineage and inhibits differentiation of TA cells to the secretory cell lineage (Figure 9) (Roulis and Flavell 2016).

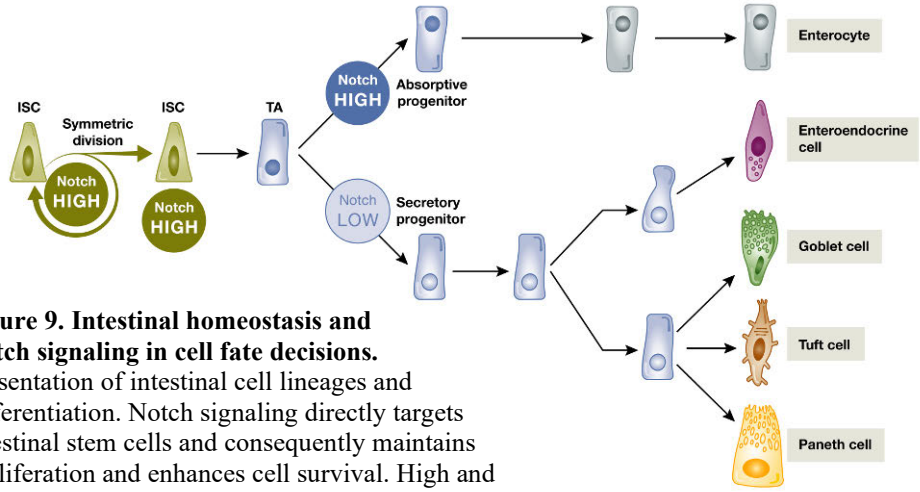

Figure 9. Intestinal homeostasis and Notch signaling in cell fate decisions. Presentation of intestinal cell lineages and differentiation. Notch signaling directly targets intestinal stem cells and consequently maintains proliferation and enhances cell survival. High and low Notch signaling results in different intestinal cell lineages. The figure is modified from (Sancho et al. 2015).

For details considering stem cell proliferation and differentiation in the colon, please see section "3.1.1 Proliferation and differentiation of colonic epithelial stem cells and progenitor cells".

Pharmacological inhibition of γ-secretase or genetic deletion of CSL in the intestinal epithelium inhibits the activity of Notch signaling and leads to the differentiation of all TA cells into cells in the secretory cell lineage. In accordance, also deletion of the Hes1 protein ends up in higher amounts of all different types of cells in the secretory lineage, and the effect of increased amounts of secretory lineage cells is greater with simultaneous deletion of Hes1, Hes3 and Hes5. TA cells not entering differentiation into the secretory cell lineage stems from NICD activating the expression mainly of the Hes1 gene whose protein product transcriptonally represses the expression of the **Math1/Atoh1** gene. Math1/Atoh1 in turn controls the potential entry of TA cells into the secretory cell lineage (Clevers 2013). Accordingly, deletion of Math1/Atoh1 results in loss of all secretory cell lineages whereas inhibition of Notch activity causes loss of Hes1 expression and initiation of Math1/Atoh1 expression in all crypt cells. Math1/Atoh1 is active only in secretory cells whereas Hes1 exists in the majority of proliferative crypt cells (van Es et al. 2005).

The cell in which Notch signaling is active, steers its neighboring cell to adopt a different final cell fate state (Figure 10) (Sancho et al. 2015). Balanced activity of Notch is crucial for the accurate maintenance of the intestinal epithelial tissue (Fre et al. 2005). Delta-Notch signaling regulating commitment of TA cells to differentiate into specific intestinal cell lineages is evolutionarily strongly conserved as this role of Notch is present in both zebrafish and mammals (Crosnier et al. 2005).

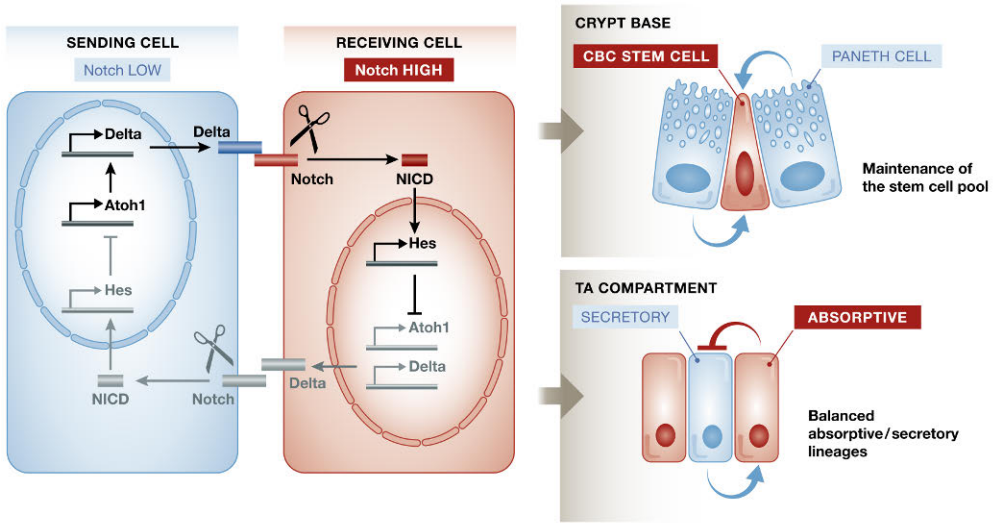

Figure 10. Notch and lateral inhibition in intestinal stem cells and TA progenitor cells. The transcriptional repressor proteins of the Hes family regulates a set of genes that have roles in the regulation of differentiation/proliferation. The mechanism for lateral inhibition is that Notch activation leads to the synthesis of the Hes protein, which represses the transcription factor Atoh1, which no longer can activate Delta transcription in the same cell. Since Notch ligands are not being produced in this cell, the cell surface Notch receptor cannot be activated in the neighboring cell driving the neighboring cell towards another cell fate. Vice versa, a cell that has low Notch activity has the capability to produce Notch ligands. The figure is adapted from (Sancho et al. 2015).

1.6 Notch in disease

Mutations that affect the Notch signaling mechanism are associated with various developmental disorders. This also highlights the pleiotropic role of Notch in the regulation of different cellular events (Bellavia et al. 2007; Komatsu et al. 2008). Both upregulated and downregulated Notch signaling are associated with diseases in the human (Gordon et al. 2008). The effects that Notch signaling mediate are dependent on the context and environment/tissue, and which Notch target genes are being activated. Therefore, whether Notch functions are oncogenic or tumor suppressive is case-specific (Figure 11) (Aster et al. 2017).

Three patterns of Notch mutations promoting cancer have been distinguished: tumors with robust gain-of-function mutations e.g. T-cell acute lymphoblastic leukemia (T-ALL), tumors with microenvironment-dependent weak gain-of-function mutations e.g. chronic lymphocytic leukemia (CLL), and tumors with loss-of-functions mutations (e.g. squamous cell carcinoma). Therefore, the cellular context is a highly determining factor of how deregulated Notch contributes to cancer development (Aster et al. 2017).

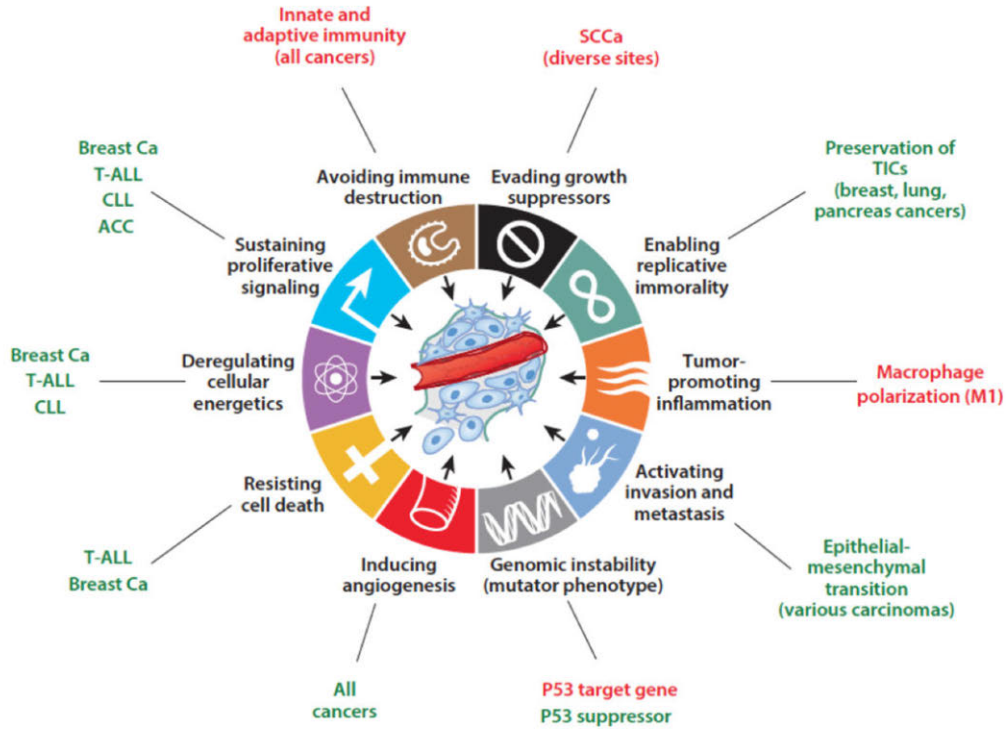

Figure 11. Cancer trademarks suggested to be impacted by Notch signaling. Oncogenic effects are shown in green, and tumor suppressor effects are shown in red. The figure is adapted from (Aster et al. 2017).

1.6.1 Notch in disease in the CNS

Notch signaling has been studied broadly in the diseased brain, more specifically in the injured and disordered neural development. It is known that several neurodegenerative diseases and brain disorders are at least partially results of aberrant Notch signaling activity. Loss-of-function mutations of Notch in the embryo and in the adult have proved the crucially important regulatory role of Notch in neurodevelopmental disorders. In the embryo, loss-of-function of Notch itself or components participating in the Notch cascade leads to too early differentiation of neural stem cells causing neurodevelopmental aberrations such as reduced survival rate and defective progenitor cell migration. Also in the adult, Notch1 loss results in loss of neural progenitor cells as the progenitor cells start to differentiate too early. Pathological conditions of neurogenesis whose development Notch is at least partially involved in include Alzheimer's disease, seizures, strokes, and gliomas which are tumors that arise in glial cells in the brain or spinal cord (Table 1) (Zhang et al. 2018b).

Table 1. Notch signaling components in pathological conditions of neurogenesis.

Condition	Effectors	Phenotype	Reference
Alzheimer's	Presenilin mutation	Defective NICD translocation	(Song et al. 1999)
	Presenilin deletion	Misexpression of Dll1 and Hes5	(Saura et al. 2004)
	Notch-presenilin interaction	Notch1 expression ↑	(Ray et al. 1999)
	APP-Numb interaction	Notch signaling ↓	(Kyriazis et al. 2008)
Gliomas	Notch mutation	Glioma formation	(Suzuki et al. 2015)
	Notch ↓ p53 ↓	Glioma growth ↑	(Giachino et al. 2015)
	Notch ↑	Drug resistance ↑	(Liau et al. 2017)
Seizures	Hes5 ↓	Status epilepticus, astrocyte dif. ↑	(Elliott et al. 2001)
	NICD ↑	Promote neuronal excitation	(Sha et al. 2014)
	Hes5 ↑	KA-induced seizure, NSC prolif. ↑	(Lugert et al. 2010)
Stroke	Notch3 mutation	CADASIL	(Louvi et al., 2012)
	NICD ↑	NPC proliferation ↑	(Wang et al. 2009)
	NICD ↑	Neuronal death ↑	(Baik et al. 2015)
	Block Notch signaling	Proliferative reactive astrocytes ↓	(Shimada et al. 2011)

Alzheimer's disease (AD) is thought to arise from γ-secretase sequentially cleaving amyloid precursor protein (APP) giving rise to amyloid beta (Aβ) assemblages. In many studies, aberrant Notch signaling has been detected in AD brains since γ-secretase also cleaves activated Notch. However, it is still very unclear if and to what extent abnormal Notch signaling is responsible for the buildup of the disease (Zhang et al. 2018b).

Mutations within proteins that participate in the Notch transduction cascade, and followed by impaired Notch signaling activity are frequently detected in malignant gliomas (Suzuki et al. 2015). Inactivation of Notch in aggressive brain tumors has been shown to resist growth of cancer cells and survival, but blocking Notch activity in gliomas enhances tumor cell proliferation resulting in the tumors transforming to a more aggressive form (Giachino et al. 2015).

Seizure which is portrayed by extensive neuron activity, harms in most cases neural stem cells and impairs neurogenesis in the hippocampus. Reduction of Hes5 expression has been

shown to occur in the hippocampal dentate gyrus after status epilepticus, also known as epileptic seizure (Elliott et al. 2001) whereas kainic acid (KA)-induced acute seizures enhance Notch signaling activity (Sha et al. 2014). Stroke, on the other hand, causes cell death in the brain and consequently leads to cognitive disruption (Zhang et al. 2018b). The role of Notch in stroke has been demonstrated for example via Notch3 whose mutation seems to be a leading cause in the inducement of the stroke in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL, a hereditary stroke disorder) (Louvi and Artavanis-Tsakonas 2012). In addition, the expression of the Hes genes is increased following ischemia (restriction in blood supply causing hypoxia) (Chen et al. 2008).

1.6.2 Notch in disease in the intestine

Acute infection, radiation injury and inflammatory bowel disease (IBD) which is a group of diseases including ulcerative colitis and Crohn's disease, are medical conditions in which damage occurs to the protective mucus barrier/layer secreted by goblet cells, or damage to the underlying intestinal epithelial tissue. This damage causes pathological inflammation in the intestine, which in turn leads to defective immune response against vast amounts of micro-organisms. Notch has a role in the development and recuperation of inflammation in the form of regulating proliferation and differentiation of epithelial cells within the intestine (Fig. 12) (Sancho et al. 2015).

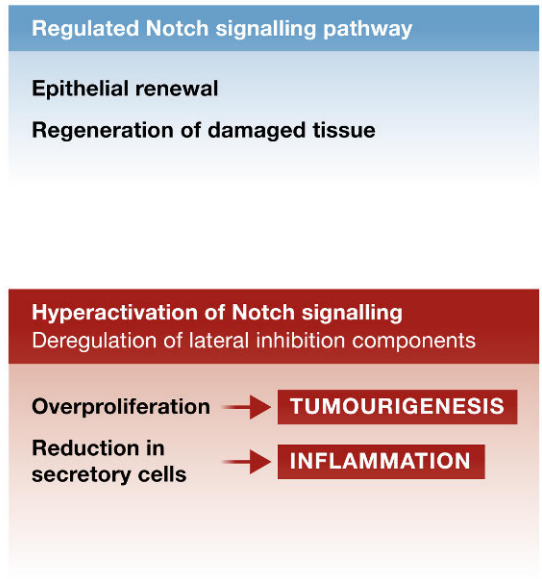

Figure 12. Accurate regulation of the Notch pathway is necessary for the correct regulation of intestinal homeostasis. If the Notch pathway regulators are defective it can lead to hyperactivation of Notch signaling activity with consequent disturbances in the intestine as shown in the figure. Also mutations of the pathway can cause its hyperactivation. The figure is modified from (Sancho et al. 2015).

Mice possessing hyperactive Notch in the form of constitutive activation of Notch do not develop secretory cells and as a consequence die in 3 days post birth (Fre et al. 2005). Reduction of secretory cells, including goblet cells secreting mucus, will lead to a weakened protective mucus barrier, which will increase the likelihood of inflammation. It has been shown that if Hes1 blocks the expression of the gene Math1/Atoh1 in excessive manners, it will be followed by goblet cell loss in ulcerative colitis (Zheng et al. 2011). This receives

support from a study showing excessive Notch activation in mouse intestine causing weak mucus secretion, which impairs recovery from colitis (Pope et al. 2014). Therefore, balanced Notch signaling is a prerequisite for avoiding intestinal dysfunctions.

Colorectal cancer (CRC) is the second most frequently occurring cancer among women and third most among men. In 2012, there were approximately 1.4 million new cases and 0.7 million deaths resulting from CRC (Aoyama et al. 2017). IBD is characterized by chronic intestinal inflammation of the gut and is known to increase the likelihood of colitis associated CRC development (Rubin et al. 2013). The pathogenesis of CRC is regulated by crosstalk between the intestinal stem cell niche and the stroma (Knight et al. 2016). The sickness is characterized by abnormal stem cell niche, resistance to apoptosis, ability to undergo inappropriate proliferation, and the ability to develop mutations that favor metastasis (Vinson et al. 2016).

Despite that no singular pathway is responsible for all the different traits in CRC, an increasing amount of studies link dysfunctional Notch signaling activity to the development of CRC. A part of the contribution of Notch to CRC development occurs from Notch cross-talking with other pathways in unfavorable ways. The regulation of both proliferation and differentiation of intestinal epithelial cells are mechanistic functions via which Notch is associated with CRC (Vinson et al. 2016). In order for CRC tumorigenesis to take place in the intestine it requires Notch1 activation carried out by beta catenin-mediated upregulation of Jagged1 (Rodilla et al. 2009). It has also been reported for instance that inhibition of Notch1 by γ-secretase inhibitors in colon cancer cells contributes to increased chemosensitivity (Meng et al. 2009) and that elevated Notch1 levels correlate with unfavorable survival rate in CRC (Chu et al. 2010). In addition, recently it was reported that overexpression of the Notch downstream target gene product Hes6 promotes metastasis via the Wnt/β-catenin signaling in CRC (Xu et al. 2018).

Generally, in the colon, Notch has been considered to be an oncogene. For instance, it was recently reported that the Notch ligand Jagged1 is constitutively processed in CRC tumors with the mutant protein K-ras (Pelullo et al. 2019). However, there is a disturbingly high amount of contradictory published data concerning the role of Notch1 in intestinal tumorigenesis and not all studies that link Notch1 to intestinal tumorigenesis report specifically increased activity of the Notch1 pathway (Sakamoto et al. 2012). For instance, it was recently reported that intestinal epithelial Notch1 protects from colorectal mucinous adenocarcinoma by preserving mucus barrier integrity. The same study highlighted that loss of Notch1 in the same conditions and environment promotes tumorigenic transformation of intestinal epithelial cells and tumor invasion (Dunkin et al. 2018). Another study has reported development of intestinal tumors as a consequence of Notch1 loss in mice (Liu et al. 2011). Additionally, Notch activated in the inflamed mucosa stimulates regeneration of the tissue. If this event is blocked for instance by deletion of CSL in mouse intestine, it will lead to chronic colitis (Obata et al. 2012). Even considering the context-dependent role of Notch, these previously mentioned studies highlight the lack of comprehension of how Notch really functions in the intestine, especially in the colon.

2. SUMO PROTEINS AS POST-TRANSLATIONAL MODIFIERS

PTMs and signaling crosstalk mediated by protein-protein interactions regulate protein function, and elucidating how Notch is modified by these events is the key aim of this thesis work. Regarding PTMs, a special focus is given to the role of cell stress-induced sumoylation in Notch regulation. The functional consequences of the modification of proteins by SUMO vary depending on the target and include the regulation of transcription, cytoplasmic-nuclear transport and DNA repair, altering protein-protein interactions and increasing protein stability (Pichler et al. 2017). Defects in the SUMO conjugation machinery are associated with the development of human disease pathogenesis, including cancer (Sarge and Park-Sarge 2009). Currently the general scientific knowledge about the SUMO-Notch interaction and its mediated biological outcomes are limited and therefore the knowledge of the regulation and the outcome of this interaction may prove to be beneficial for improving human health.

2.1 Ubiquitin-like proteins

One of the research areas in cell biology focuses on identifying and analyzing proteins that are modified by the protein ubiquitin or ubiquitin-like proteins (UBLs) (Downes et al. 2006). All eukaryotic cells express the strongly evolutionarily conserved ubiquitin consisting of 76 amino acids. Of the 76 amino acids only three residues are different between human and yeast (Kurepa et al. 2003; Pickart and Eddins 2004; Pickart and Fushman 2004). Similar to ubiquitin, all ubiquitin-like proteins follow a similar multienzyme conjugation system in which the target protein is bound on one or several lysine residues (Johnson and Blobel 1997; Wilson and Heaton 2008).

Ubiquitin and ubiquitin-like proteins possess high similarities in their structures and conjugation pathways, but differ clearly in the cellular functions they mediate (Fuchs and Neuwirtová 2006). Ubiquitination is most often described as a modification which marks proteins for degradation by the 26S-protesome (Hershko and Ciechanover 1998; Chen and Sun 2009). Other cellular outcomes mediated by ubiquitins include alterations in transcription, immune response, apoptosis and the cell cycle (Schnell and Hicke 2003; Winget and Mayor 2010). Ubiquitin is thereover involved in DNA damage repair. The nuclear proteins Proliferating-cell nuclear antigen (PCNA) and Fanconi's anaemia complementation group D2 (FANCD2) are targets of ubiquitination as a consequence of damage in the DNA sequence. These proteins are normally located close to the damage and can be either mono- or polyubiquitated (Selvarajah and Moumen 2010).

The amount of ubiquitin molecules being attached to the substrate influences the outcome of ubiquitination. A target protein for ubiquitination can be bound by one ubiquitin molecule (monoubiquitination) and in other cases several separate individual ubiquitins bind to separate target lysines on the substrate (multiubiquitination) (Hochstrasser 2006). Monoubiquitination has been shown to regulate a variety of cellular processes such as DNA repair and receptor endocytosis (Ikeda and Dikic 2008). Ubiquitin can even more commonly ubiquitinate itself. In this case, every ubiquitin molecule is covalently attached

to a specific lysine residue of the previous ubiquitin within the ubiquitin chain. The polyubiquitins can bind to the target protein as a chain of ubiquitins (polyubiquitination) (Bassermann et al. 2014).

The outcome of ubiquitination is also affected by on which lysine residue on ubiquitin the chain is being formed (Figure 13). Ubiquitin chains bound on K48 mark the substrate for proteasomal degradation. Chains attached to K63 are normally associated with non-proteasomal functions including endocytosis of membranes (Hicke and Dunn 2003; Tenno et al. 2004; Varadan et al. 2004). In 2009 it was discovered by masspectrometric studies that both K48 and K63 linked ubiquitin chains can be present in 26S-proteasome bound polyubiquitinated substrates *in vivo* (Saeki et al. 2009). At least four ubiquitins (tetraubiquitin) must form a chain with each other in order for the substrate to be effectively degraded proteasomally via ubiquitination (Thrower et al. 2000).

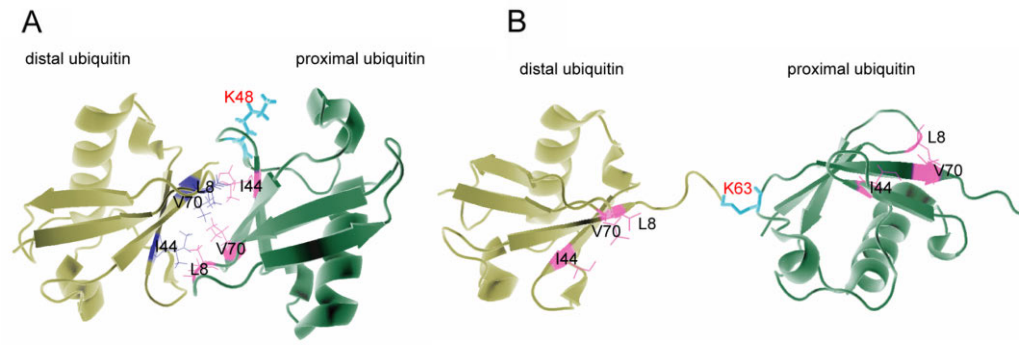

Figure 13. The outcomes of ubiquitination are characterized by for instance via which lysine residues the chain formation occurs. The most studied polyubiquitin chains are bound to each other via K48 and K63. **A)** K48-linked diubiquitins mostly form a closed formation. **B)** K63-linked diubiquitins accomplish an open conformation. The figure is modified from (Li and Ye 2008).

The similarities between different ubiquitin-like proteins are not based in most parts on sequence homology, but rather on specific features in their structures. The carboxyl group of the C-terminal glycine residue binds to the substrate's target lysine residue via isopeptide bond formation. The conjugation can have an impact on the substrate's cellular localization, enzymatic activity, or mediate or block the binding of the substrate to a third molecule (Herrmann et al. 2007). On top of this there are connections between these different conjugation pathways since some proteins can be modified by several different types of ubiquitin-like proteins (Kerscher et al. 2006). Dysregulation of ubiquitin-like proteins can lead to different diseases including several types of cancers and neurodegenerative diseases, please see section "2.5.1 Sumoylation in disease".

2.2 The SUMO family

The PTM mediated by small-ubiquitin like modifier (SUMO) proteins has grown to be considered as one of the main protein modifications in eukaryotes (Flotho and Melchior 2013). SUMO which was identified in 1995 in *Saccharomyces cerevisiae* exists in all

eukaryotes (Müller et al. 2004). SUMO1 conjugation of the first discovered SUMO substrate Ran-GTPase-activating protein (RanGAP1) results in the translocation of the substrate from the cytosol to the nuclear pore complex (Matunis et al. 1996; Mahajan et al. 1998) where RanGAP1 interacts with RANBP2/NMP358, which is a necessity for the import of proteins into the nucleus (Saitoh et al. 1997).

SUMO2 and SUMO3 are most often referred to as simply SUMO2/3 because of their high sequence similarity which is 95%. The sequence identity between SUMO1 and SUMO2/3 is only 45% (Zhang et al. 2008). The 101 amino acid long SUMO1 isoform is 18% sequentially similar to ubiquitin (Johnson and Gupta 2001; Müller et al. 2004). SUMO1-3 are expressed in all eukaryotic cells while the less studied SUMO4 has so far been observed in pancreatic, immune and renal cells (Baczyk et al. 2017). SUMO4 contains a C-terminal proline residue, which will prevent its conjugation (Guo and Henley 2014). For further details concerning SUMO conjugation, please see section "2.3 The SUMO conjugation pathway". In cells during physiological conditions, SUMO4 undergoes degradation in a fast rate, which is not the case for SUMO1-3. Relatively recently it was reported that SUMO5, a potential new SUMO isoform, mediates the growth and disruption of promyelocytic leukemia nuclear bodies (PML-NBs). However, the study does not present the existence of endogenous SUMO5 (Liang et al. 2016).

The SUMO proteins are mainly nuclear proteins and resemble ubiquitin only from a mechanistic perspective regarding conjugation (Jürgen Dohmen 2004). SUMO1, SUMO2 and SUMO3 also have a similar globular structure as ubiquitin (Figure 14). The mentioned proteins possess remarkable similarities and contain a characteristic ββαββαβ fold (Bayer et al. 1998). The N-terminal arms that are conserved in the SUMO proteins do not exist in ubiquitin and other UBLs. The arms contribute to the formation of SUMO polymer chains (Matic et al. 2008b). In budding yeast *Saccharomyces cerevisiae*, the SUMO homolog is called Suppressor of mif two 3 (SMT3) (Tang et al. 2008). The nematode *Caenorhaditis elegans* also has only one SUMO homolog, SMO-1 (Surana et al. 2017).

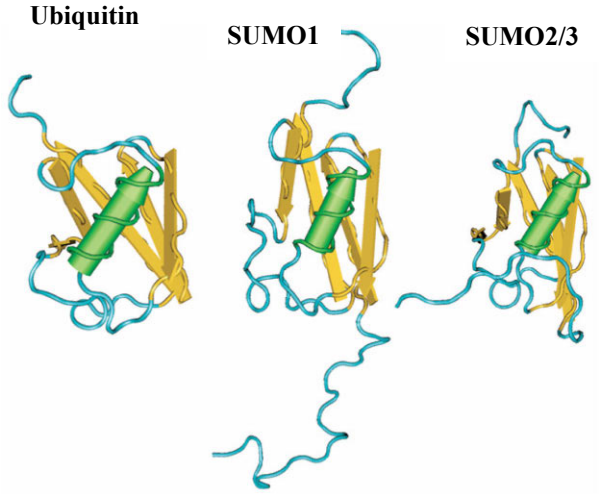

Figure 14. The three-dimensional structures of ubiquitin, SUMO1 and SUMO2/3. Both the SUMO proteins and ubiquitin contain a ββαββαβ fold, but in comparison to the SUMO proteins, ubiquitin is lacking an N-terminal tail. The C-terminal part of SUMO and ubiquitin is modified during processing of the immature SUMO protein whereafter two glycine residues which are necessary for conjugation, are being displayed. The figure is modified from (Martin et al. 2007).

In some cases, direct crosstalk between ubiquitination and sumoylation may appear when SUMO and ubiquitin compete for the same binding site, i.e. the same amino acid residue of the same proteins thus inhibiting the other PTM and the biological outcome it mediates (McManus et al. 2017). Co-regulation of sumoylation and ubiquitination levels takes place for instance on deubiquitinating proteins (Lamoliatte et al. 2017). In 2009 results showed that in human cells SUMO can function as a signal for the recruitment of E3 ubiquitin ligases resulting in the ubiquitination and degradation of the sumoylated protein (Geoffroy and Hay 2009). Later it was confirmed that SUMO-targeted ubiquitin ligases (STUbl), indeed recognize sumoylated proteins and by ubiquitination target them for degradation by the proteasome (Sriramachandran and Dohmen 2014). For instance, polysumoylation of the nuclear receptor NR4A1 triggers its ubiquitination by the SUMO-targeted ubiquitin ligase RNF4, leading to the degradation of NR4A1, which in turn regulates macrophage cell death (Zhang et al. 2017).

SUMO1 and SUMO2/3 mediate their functions by related mechanisms, but SUMO1 and SUMO2/3 still have substrates and functions that differ from each another (Zhang et al. 2008). Quantitative proteomics experiments performed revealed that out of 44 identified SUMO substrates, nine were modified by both SUMO1 and SUMO2/3 (Vertegaal et al. 2006). A year prior it had been demonstrated that from 144 newly discovered substrates, 27 were modified by both SUMO1 and SUMO2/3. These results not only give support to the concepts of different SUMO proteins partly mediating different functions, but also highlight that some substrates are able to be conjugated by different SUMO isoforms. In addition, SUMO1 and SUMO2/3 bind to different substrates depending on their cellular localization (Rosas-Acosta et al. 2005). Serine 2 is phosphorylated in SUMO1 increasing the complexity of sumoylation since SUMO therefore can operate as a modified modifier. Despite that SUMO2 and SUMO3 are sequentially 95% identical, only SUMO3 contains a serine residue at position 2 (Matic et al. 2008b).

2.3 The SUMO conjugation pathway

Conjugation of SUMO to a specific lysine residue on the SUMO substrate occurs through an enzymatic signaling mechanism which in most parts resembles ubiquitin's corresponding conjugation mechanism. Despite the similarities between these two conjugation machineries they are distinct from each other (Yang et al. 2008; Wilkinson and Henley 2010). Sumoylation is mediated by SUMO-specific activating E1-, conjugating E2- and ligating E3-enzymes (Pichler et al. 2017). Sumoylation is a process that is rapidly reversed by desumoylating proteins and usually several cycles of conjugation/deconjugation are required for sumoylation to mediate a specific function (Bossis and Melchior 2006; Wilkinson and Henley 2010). SENPs are able to deconjugate the SUMO proteins (desumoylation) from their target proteins (Figure 15) (Kumar and Zhang 2015). In sumoylation there is one conjugating enzyme, a limited amount of ligating enzymes and seven SENPs (Shen et al. 2009). Since many sumoylated lysines are also targets for other PTMs, such as ubiquitination and acetylation, crosstalk occurs between SUMO and other PTMs.

All ubiquitin-like proteins contain a C-terminal glycine residue which is needed for conjugation to the final target protein. In the sumoylation conjugation pathway the

immature SUMO protein is first processed by SENP1 or SENP2 proteins in order to expose a C-terminal di-glycine motif on SUMO (Sarge and Park-Sarge 2009; Hickey et al. 2012).

After SUMO has undergone proteolytic maturation in the form of being cleaved by SENP, the heterodimer SUMO-activating enzyme subunit 1/SUMO-activating enzyme subunit 2 (SAE1/SAE2), also known as simply E1, catalyzes the adenylation of the SUMO protein's COOH group. The adenoviral protein Gam1 is in some cases capable of blocking the activity of SAE1/SAE2 and thus halt the sumoylation cycle (Boggio et al. 2004). Following the adenylation of the SUMO protein's COOH group by SAE1/SAE2, an ATP-dependent thioester bond takes place between the SH group of the catalytic cysteine in SAE2 and the SUMO protein's COOH group (Wang et al. 2007a).

Post-activation of SUMO, the protein is being transferred to the conjugating enzyme E2, normally referred to as Ubiquitin conjugating enzyme 9 (Ubc9). Despite its name, Ubc9 is the only known protein being able to conjugate SUMO to its target protein. Ubc9 contains a catalytic cysteine residue 93 with which SUMO also forms a thioester bond (Macauley et al. 2006; Wang et al. 2007a). This event is followed by Ubc9 catalyzing the binding of SUMO to one or several target lysine residues on the substrate. The catalysis happens either directly or through E3-mediated reactions (Bernier-Villamor et al. 2002). SUMO binds to its target protein through an isopeptide bond between the SUMO protein's free C-terminal glycine and the ε-amino group of the target lysine on the SUMO substrate (Melchior 2000). The heterodimer activating complex SAE1/SAE2, and the conjugating enzyme Ubc9 are distinct from the ones that participate in the conjugation pathways of other ubiquitin-like proteins (Wang 2009). Although Ubc9 is homologous with ubiquitin-like conjugating E2-enzymes it is still SUMO-specific and cannot form a thioester bond with ubiquitin (Desterro et al. 1997).

The interaction between Ubc9 and most SUMO target proteins is not stabile enough for efficient conjugation, which is why strong sumoylation often requires the participation of E3 ligases (Gong et al. 1999). These ligases increase the sumoylation efficiency by interacting both with Ubc9 and the SUMO substrate (Sarge and Park-Sarge 2009). However, E3 ligases are not always required. Already in 1999 it was shown that only in the presence of SAE1/SAE2, Ubc9 and ATP, SUMO1 is efficiently conjugated to its substrate inhibitor of nuclear factor-κB (IκBα) (Desterro et al. 1999).

Many different SUMO E3 ligases have been identified. Most of these contain a Siz/PIAS (SP)-ring motif (Zuo and Cheng 2009). The members in the Siz-PIAS (protein inhibitors of activated STAT [signal transduced and activator of transcription]) family are PIAS1, PIASXα, PIASXβ, PIAS3, PIASy and Nse2Mm21 (Guo and Henley 2014). The PIAS proteins originally received their name from their ability to inhibit STAT factors, but since they are able to participate case specifically in the SUMO conjugation pathway it became clear that PIAS proteins have a broader role in transcriptional and cellular regulation (Palvimo 2007). Other classes of SUMO E3 ligases except for the Siz-PIAS family are the Ran binding protein 2 (RANBP2) family and the ZNF451 family (Pichler et al. 2017). Additionally, class II HDACs have been shown to possess SUMO E3 ligase activity in the sumoylation of various substrates (Garcia-Dominguez and Reyes 2009).

SENPs are specific isopeptidases that can hydrolyze the isopeptide bond between SUMO and the SUMO substrate leading to detachment of SUMO from the SUMO target protein (Yeh et al. 2000; Boggio and Chiocca 2005). Members of the SENP family include SENP1-3 and SENP5-8, although SENP8 does not exhibit specificity for SUMO, but for the ubiquitin-like Nedd8 (Kunz et al. 2018). The SENP proteins identified in human are classified into three different groups based on sequence and main subcellular localization (Mendes et al. 2016). A very limited amount of other desumoylating proteins have been identified. This rare group includes Ubiquitin-specific protease-like 1 (USPL1) (Schulz et al. 2012), DeSumoylating Isopeptidase 1 (DeSI-1) and DeSumoylating Isopeptidase 2 (DeSI-2) (Shin et al. 2012) (Table 2). After desumoylation, the SUMO proteins can once again be attached to target proteins (Sarge and Park-Sarge 2009).

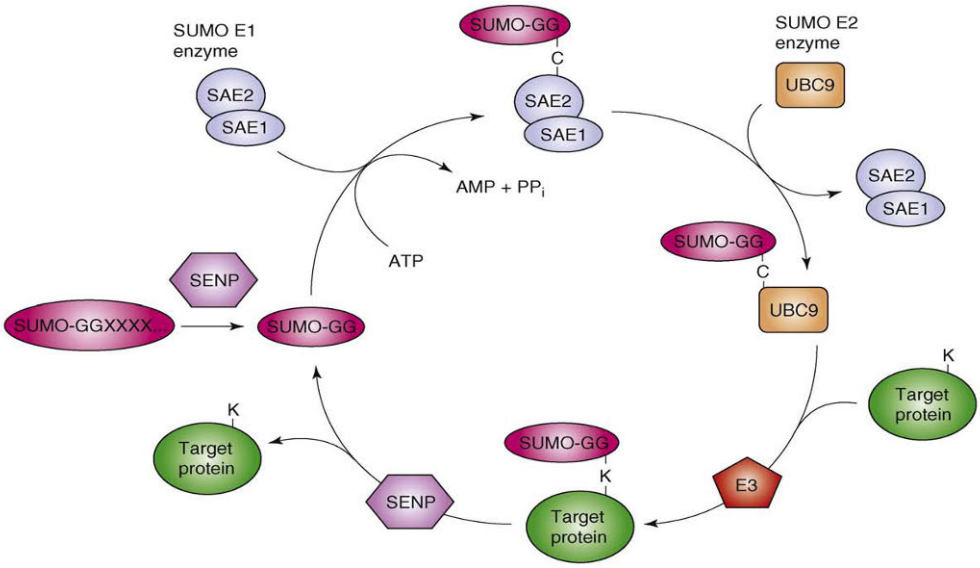

Figure 15. Conjugation of SUMO to its substrate. The conjugation pathway is initiated by SENP proteins cleaving SUMO to its mature form. From the SUMO protein's C-terminus a SENP protein cleaves off 4, 11 or 2 amino acids depending on whether the isoform to be processed is SUMO1, SUMO2 or SUMO3. The processed SUMO protein is thereafter activated by conjugation to the E1 heterodimer SAE1/SAE2 after which SUMO is bound to the E2 enzyme Ubc9. Finally, SUMO is conjugated to its substrate via an isopeptide bond between the C-terminal glycine on SUMO and the ε-amino group of the target lysine on the substrate. The binding efficiency is case specifically strengthened by E3 ligases that interact both with Ubc9 and the substrate. Desumoylating proteins can end the sumoylation reaction after which SUMO can be bound to other target proteins. The figure is adapted from (Sarge and Park-Sarge 2009).

Table 2. Localization and actions of SUMO proteases. Table is adapted from (Henley et al. 2014).

SUMO Protease	Main Cellular Location	SUMO Selectivity	Cleavage of ProSUMO	Removal of SUMO From Target Substrates
SENP1	Mainly nucleus and subnuclear structures but also present in cytoplasm and other extranuclear compartments	SUMO1 & SUMO2/3	Yes SUMO1 & SUMO2	Yes
SENP2	Mainly nucleus and subnuclear structures but also present in cytoplasm and other extranuclear compartments	SUMO2/3 > SUMO1	Yes SUMO1 & SUMO2	Yes
SENP3	Nucleolus as well as extranuclear compartments including mitochondria	SUMO2/3	ND	Yes
SENP5	Nucleolus as well as extranuclear compartments including mitochondria	SUMO2/3	Yes SUMO3	Yes
SENP6	Nucleoplasm	SUMO2/3 chains	No	Yes, preferentially SUMO2/3 chains
SENP7	Nucleoplasm	SUMO2/3 chains	No	Yes, preferentially SUMO2/3 chains
DeSI1	Nucleus and cytoplasm	SUMO1 & SUMO2/3	Possibly	Yes
DeSI2	Nucleus and cytoplasm	ND	No	ND
USPL1	Cajal bodies in nucleus	SUMO2/3 > SUMO1	Possibly	Yes

2.4 Sumoylation specificity

2.4.1 Sumoylation in the consensus motif

A consensus sumoylation site was discovered when the lysine residues in only a handful of early identified SUMO target proteins where mapped. These SUMO target proteins included RanGAP1, p53, c-Jun, promyelocytic leukemia (PML), Sp100, and IκBα (Geiss-Friedlander and Melchior 2007). It turned out that often the lysines being targets for SUMO are located in a minimal consensus sequence, in the tetrapeptide ΨKxE/D. In the consenus sequence, Ψ represents a large hydrophobic amino acid, K represents the acceptor lysine, x represents any amino acid, E represents glutamate and D represents aspartate (Rodriguez et al. 2001). The majority of the SUMO substrates contain the sequence. Evolutionary conservation of the consensus sequence indicated an important role for the sequence in SUMO conjugation (Sampson et al. 2001). The identification of a consensus sequence was remarkable since no consensus sequences had been found for ubiquitin. The reason may at least partly lie in that over 20 ubiquitin conjugating enzymes have been discovered, but only one SUMO conjugating enzyme. Ubc9 recognizes the consensus sequence only if it is part of an extended loop like in RanGAP1, or is present in an unstructured area like in the transcription factor ETS1, or the N-terminus of SUMO2/3. Ubc9 is not able to recognize the consensus sequence in stable helical structures (Geiss-Friedlander and Melchior 2007). The interaction between the SUMO consensus site and the Ubc9 catalytic cleft is not stabile enough on its own as such, but requires stabilization either by participation of SUMO E3 ligases, or by additional co-factors of interfaces (Pichler et al. 2017). SUMO2/3/4 contain the SUMO consensus sequence, but SUMO1 does not (Tang et al. 2008). Several extended variants have been described, and atypical sites with little similarity to any of the so far identified sequences that help predict sumoylation sites also exist (Yang and Chiang 2013).

2.4.2 Sumoylation in extended motifs

The SUMO consensus sequence is present in over a third of all characterized proteins. To increase the specificity and to ease the identification of new SUMO substrates many

attempts in mapping residues around the traditional SUMO consensus sequence have been made. In 2006, a new determinant was found: negatively charged amino acid-dependent sumoylation motif (NDSM) consisting of clusters of acidic residues shortly after the consensus sequence. The acidic residues increase the sumoylation efficiency by binding with electrostatic interactions to basic residues on Ubc9 thus enhancing the binding efficiency between the substrate and Ubc9 (Figure 16). Using the NDSM in searches, the amount of potential SUMO substrates can be decreased to 15% compared to 35% when only the consensus sequence is used (Yang et al. 2006).

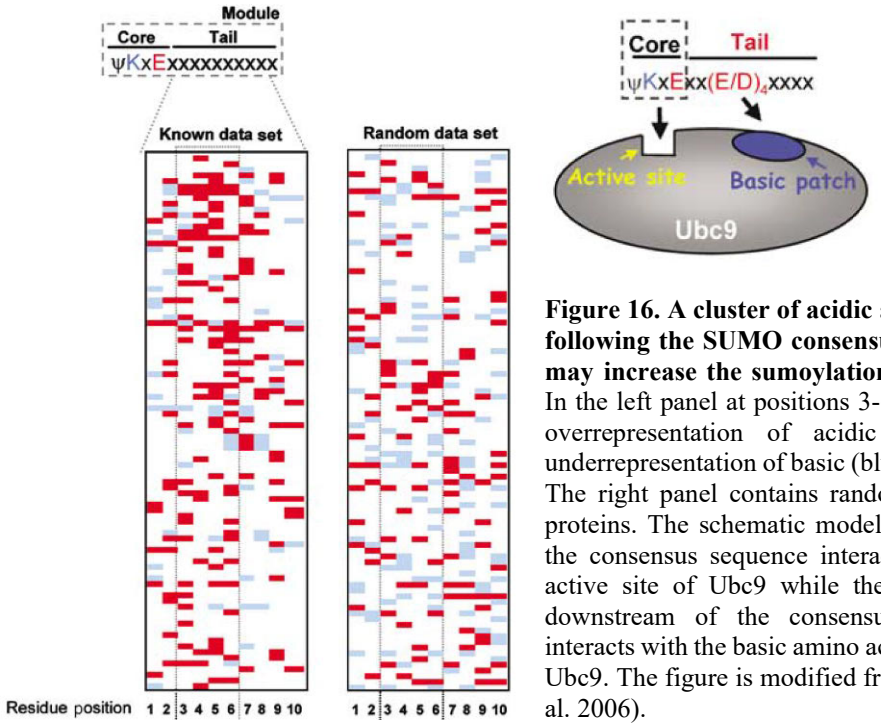

Figure 16. A cluster of acidic amino acids following the SUMO consensus sequence may increase the sumoylation efficiency. In the left panel at positions 3-6 there is an overrepresentation of acidic (red) and underrepresentation of basic (blue) residues. The right panel contains randomly picked proteins. The schematic model shows how the consensus sequence interacts with the active site of Ubc9 while the tail region downstream of the consensus sequence interacts with the basic amino acid cluster of Ubc9. The figure is modified from (Yang et al. 2006).

Except for NDSM, many other extended/modified sumoylation motifs have been identified in SUMO target proteins. Phosphorylation-dependent sumoylation motif (PDSM) consisting of the sequence ΨKxExxSP regulates phosphorylation-dependent sumoylation of many SUMO substrates, mainly transcription factors. Heat shock factor 1 (HSF1) is sumoylated in K298 as a consequence of phosphorylation in S303. Other known transcription factors undergoing phosphorylation-dependent sumoylation include erythroid transcription factor 1 (GATA-1) and myocyte-specific enhancer factor-2 (MEF-2) (Hietakangas et al. 2006). Some proteins such as RanGAP1 and ZBTB1 are sumoylated via their hydrophobic cluster sumoylation motif (HCSM). In this case, the sumoylation takes place on a lysine residue within the basic SUMO consensus motif, but the target lysine residue is preceded by at least three hydrophobic amino acids instead of the usual single amino acid. In addition, some SUMO target proteins are conjugated by SUMO in an inverted SUMO consensus motif E/DxKΨ (Matic et al. 2010).

2.4.3 Non-consensus sumoylation and other determinants

Not all SUMO target lysine residues are located inside consensus sequences. Other specificity determinants exist and have been identified for sumoylation. These include appropriate presentation of the sequence of the substrate, and the cellular environment dependent on the cellular location (Palvimo 2007). Some SUMO substrates are sumoylated at both consensus sequences and non-consensus lysines (Johnson 2004). Upon cell stress, more lysines are sumoylated at non-consensus sites compared to the consensus site (Pichler et al. 2017).

Regulation of sumoylation occurs mainly through alterations in the activity of the SUMO proteins binding to their target proteins. These alterations can be directly modified by cellular stimuli, such as hypoxia and reactive oxygen species (ROS). Regulation of SUMO conjugation occurs also through alterations of expression of proteins that participate in the sumoylation conjugation pathway. Also crosstalk between sumoylation and other PTMs can case specifically regulate sumoylation (Liu and Shuai 2008). Phosphorylation can function as a determinant for sumoylation activity though PDSM (Hietakangas et al. 2006). In some cases, there can be competition between SUMO proteins and ubiquitin proteins to the same conjugation site. In these cases SUMO can negatively regulate the substrate from degradation mediated by ubiquitin (Ulrich 2005). However, in some cases, such as with HIF-1α, sumoylation can target a protein for degradation through the ubiquitin-proteasome pathway (Cheng et al. 2007). Also acetylation and methylation can compete with sumoylation for the same target sites (Macauley et al. 2006).

SUMO conjugation where the consensus sequence is recognized directly by Ubc9 takes place without the participation of non-covalent bindings (Zhu et al. 2008). In some cases, SUMO conjugation takes place via a SUMO-interacting motif (SIM) on the substrate (Seu and Chen 2009). The SIM was discovered in 2000 with yeast two-hybrid screening (Minty et al. 2000) and a large amount of proteins contain this motif that non-covalently interacts with SUMO (Figure 17) (Zhu et al. 2008). Two-hybrid screening was used to distinguish between covalent and con-covalent interactions with SUMO. Proteins that can associate with SUMO in the absence of the C-terminal di-glycine interact with SUMO specifically via non-covalent bindings (Kerscher 2007). SIM contains a hydrophobic core which is surrounded by acidic amino acids or serine residues (Meulmeester et al. 2008). It has been shown that the function of the SIM in for instance the transcriptional co-repressor Daxx protein is a prerequisite for the transrepression of several sumoylated transcription factors including glucocorticoid receptor (Lin et al. 2006). Later it was demonstrated that Daxx contains two SIM motifs. Both motifs are located in their own terminus of the protein, and both motifs can independently of the other motif associate with SUMO. Double mutation of K17/K733 both of which are located in their own SIM, resulted in that Daxx no longer interacts with SUMO (Santiago et al. 2009).

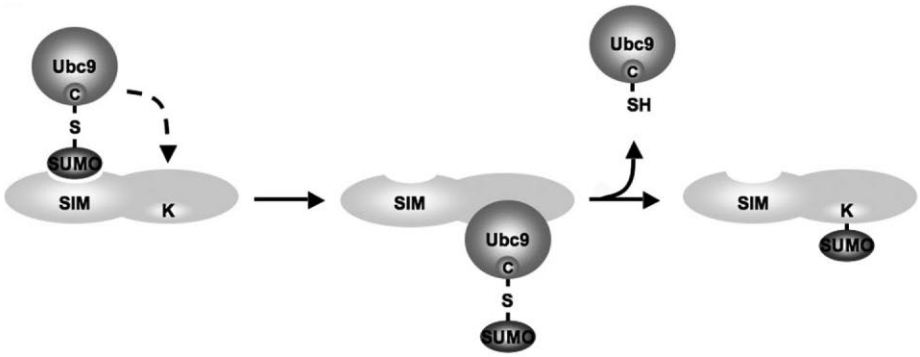

Figure 17. Sumoylation can in some cases be mediated by non-covalent SUMO binding. A SIM containing protein is being bound by a SUMO protein which is covalently bound to the active cysteine of Ubc9. The binding increases the local concentration of Ubc9 in the proximity of the SUMO substrate. Therefore, Ubc9 can easier recognize the target lysines that are not necessarily located in a consensus sequence. Thereafter SUMO is conjugated covalently to the substrate and Ubc9 is set free. The different SUMO isoforms are recognized by SIM through the different affinities that SIMs have for different SUMO isoforms. The figure is modified from (Zhu et al. 2008).

A high-throughput screen for SUMO2 sites identified several phosphorylation and acetylation sites close to the SUMO2 sites in a set of more than 1600 proteins (Hendriks et al. 2014). Current methodologies to search for new SUMO substrates and sites rely mainly on enrichment of SUMO modified peptides, and thus finding new endogenous SUMO substrates is experimentally demanding (Tammsalu et al. 2014). However, a high-throughput method combining peptide immunocapture and quantitative proteomics was developed, which managed to identify 295 SUMO1 and 167 SUMO2 sites on endogenous substrates (Impens et al. 2014).

2.4.4 SUMO chains

Like ubiquitins, also SUMO proteins are able to form polymeric chains on many of their substrates and similar to ubiquitins the chain formation is reversible also among SUMO proteins (Tatham et al. 2001; Vertegaal 2010). Ubc9 has the capability to recognize the consensus sequence on its substrate regardless if the substrate is a SUMO protein or not. SUMO2 and SUMO3 have a consensus lysine at position 11 while SUMO1 lacks the consensus lysine residue (Figure 18). Ubc9 catalyzes the chain formation of free SUMO2/3 *in vitro* via K11, but chains are formed *in vitro* also via non-consensus lysine residues (Ulrich 2008). Also, chains of SUMO1 are formed *in vitro* via the non-consensus lysine residues 7, 16 and 17, but SUMO1 form polymeric chains less efficiently than SUMO2/3 (Pichler et al. 2002; Pedrioli et al. 2006; Ulrich 2008). This is partly because of the lack of K11 in SUMO1, but also because SUMO1 lacks K5, which has been shown to undergo SUMO conjugation in SUMO2/3 (Mohideen and Lima 2008; Vertegaal 2010).

All different SUMO paralogs are able to form chains with each other both *in vitro* and *in vivo*. This takes place by a SUMO protein being attached to the consensus K11 on

SUMO2/3. If the protein that attaches to SUMO2/3 happens to be SUMO1, the chain cannot be further extended because of the lack of K11 on SUMO1 (Matic et al. 2008a).

The chain formation of SUMO2/3 has been observed to increase markedly during cellular stress (Golebiowski et al. 2009), and E3 ligases have been observed to increase the efficiency of SUMO polymerization *in vitro* (Pichler et al. 2002).

Although E1-E3 ligases have the capability to assemble SUMO chains, more detailed mechanisms that specifically elongate these chains have been less understood. E4 elongases are unable to assemble the chain, but are able to elongate the chain (Eisenhardt et al. 2015). *In vivo*, the detection of SUMO chains can be challenging since the chains might be difficult to distinguish from multisumoylated substrates and from large monosumoylated substrates (Ulrich 2008).

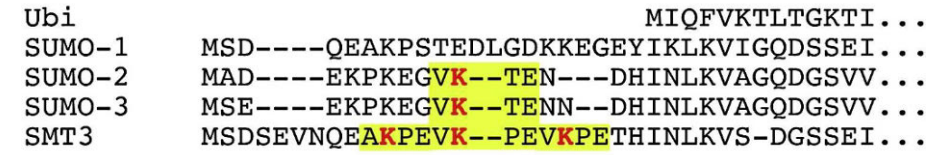

Figure 18. Chain formation in SUMO. A) A sequence comparison of ubiquitin's and the different SUMO isoforms' N-termini. The consensus sequence is shown in yellow and the acceptor lysine in red. The figure is modified from (Ulrich 2008).

2.4.5 Protein group sumoylation

The physiological consequences of sumoylation are difficult to assess, since SUMO-mediated outcomes often depend on modification of several proteins which function collectively (Jentsch and Psakhye 2013). More specifically, mutation of a sumoylation target lysine residue may not be reflected in any form of physiological alterations since these physiological outcomes may be more dependent on broader multisumoylations of interrelated protein complexes (Tammsalu et al. 2014). This may not even be limited to a single SUMO target lysine residue. It is perhaps even plausible that in some cases blockage of all sumoylation sites within a protein may not be detectable in terms of physiological effects (Jentsch and Psakhye 2013). This thinking represents the "spray" theory within the SUMO field in which spatially related clusters of SUMO protein substrates are sumoylated simultaneously (Guo and Henley 2014). This is supported by a study in which 60% of the novel SUMO target proteins that were identified, are part of larger functional protein groups (Hendriks et al. 2014). This hypothesis may be potentially used as a platform for further studies regarding how networks of proteins undergo simultaneous SUMO conjugation as a cause of cell stress (Guo and Henley 2014). SUMO target proteins within the Notch pathway include Notch1, MAML1, and also the target gene protein product Hes1 (Poulin et al. 2005; Lindberg et al. 2010). Since Notch1 and MAML1 interact on the NICD1 transcriptional complex, it is possible that sumoylation regulates the pathway on a broader level through synchronous targeting of several proteins within the pathway.

2.5 SUMO in biological functions

The consequences of sumoylation are case-specific and depend on which substrate is being modified. The first studies indicated that sumoylation mainly takes place in the nucleus, but later an increasing amount of non-nuclear SUMO substrates were identified (Zhao 2007).

Sumoylation regulates its substrate in most cases by influencing the substrate's activity or interactions with other proteins (Zhao 2007). Translocation of SUMO substrates is also an essential widely occurring outcome of sumoylation. By using extensive mutagenesis, it was shown that the receptor insulin-like growth factor 1 (IGF-1R) undergoes sumoylation in the lysines 1025, 1100 and 1125 causing the translocation of IGF-1R to the nucleus. IGF-1R was the first receptor tyrosine kinase (RTK) to be detected to be sumoylated (Sehat et al. 2010). Sumoylation of IGF-1R increases cell proliferation in acute myeloid leukemia (Zhang et al. 2015) and promotes cell cycle progression (Lin et al. 2017). Additionally regarding RTK sumoylations, the RTK ERBB4 intracellular domain, cleaved by γ-secretase from its full-length receptor, is sumoylated leading to increased nuclear sequestration (Sundvall et al. 2012) and RTK dimerization (Knittle et al. 2017).

It is challenging to predict exact molecular consequences that SUMO mediates for a specific SUMO target protein. The reason lies in the fact that sumoylation mediates such a broad range of biological outcomes (Geiss-Friedlander and Melchior 2007). At a molecular level sumoylation can affect its target protein in three different ways depending on the case and therefore affect its interactions with other proteins (Figure 19).

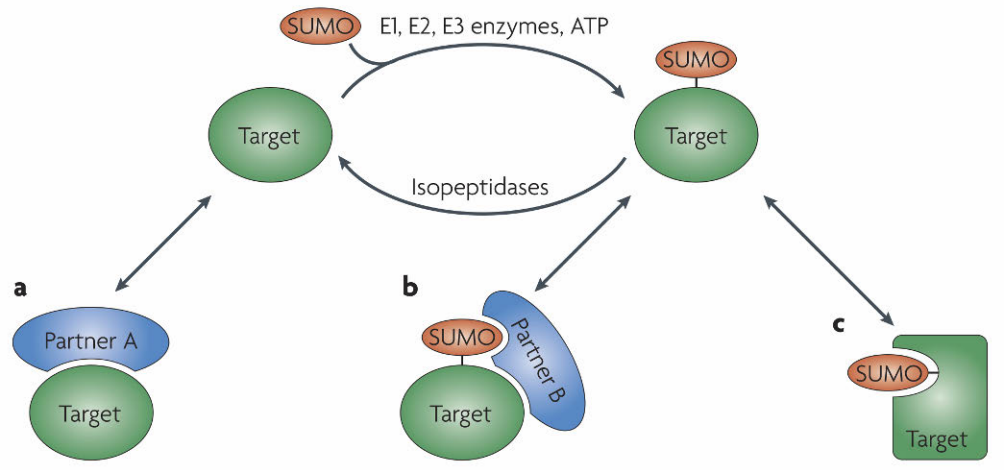

Figure 19. At a molecular level, there are three different consequences for a SUMO modified protein. A) Sumoylation can block the interaction between the target protein and its binding partner and in that case the interaction takes place only in the absence of sumoylation. **B)** Sumoylation can provide a binding site for a co-binding partner protein. **C)** Sumoylation can result in the restructuring of the substrate's conformation. The figure is adapted from (Geiss-Friedlander and Melchior 2007).

Transcriptional regulation is one of the most common outcomes of sumoylation. Examples of early identified sumoylated transcription factors include Sp3, c-Jun and c-Myb (Verger et al. 2003). Binding of a SUMO protein to transcription factors do not have universal consequences regarding transcription as SUMO can indirectly both activate and inhibit gene transcription depending on the target protein (Girdwood et al. 2004; Ehninger et al. 2007). However, it was already known over a decade ago that in most cases SUMO conjugation to transcription factors leads to repression of transcription (Girdwood et al. 2004; Gill 2005).

Sumoylation has been shown to affect for instance protein stability and response to cell stress (Guo and Henley 2014). Sumoylation as well as ubiquitination and phosphorylation have an impact on the cell cycle. Total inhibition of sumoylation in mammalian cells causes halting of the cell cycle's prometaphase. This is a result from the fact that the microtubule motor protein, centromerprotein E (CENP-E) which is normally conjugated by SUMO2/3 is no longer sumoylated. Sumoylation of CENP-E is needed for its translocation to the kinetochores (Zhang et al. 2008). The kinase Mps1 which is a target for sumoylation during the cell cycle has a role in the regulation of the spindle assembly checkpoint and mediates the correct attachment of microtubules to chromosomes during mitosis. By conjugating to Mps1, SUMO mediates kinetochore localization of Mps1 and therefore has an impact on normal mitotic progression (Restuccia et al. 2016).

From a Notch perspective, sumoylation has also been shown to regulate cell fate specification during development, and sumoylation can trigger various differentiation processes (Lomelí and Vázquez 2011). Halting differentiation during development causes organ failure leading to embryonic lethality. Inhibiting sumoylation at embryonic stages of development is either lethal or results in serious dysfunctions and abnormalities. The sumoylation status of many transcription factors and chromatin remodeler proteins that regulate gene expression, directs accurate progression of development and cell differentiation (Deyrieux and Wilson 2017). Notch1 adds to the body of the SUMO target proteins that steer development and differentiation (Study I).

2.5.1 Sumoylation in disease

Defects in the SUMO conjugation machinery are associated with many human pathologies including different forms of cancers (Flotho and Melchior 2013). Sumoylation maintains repair of DNA damage and maintains also on a broader level genome integrity (Seeler et al. 2007; Sekiyama et al. 2010). It seems that increased sumoylation predominantly is a cellular protective response (Guo and Henley 2014). Therefore too excessive sumoylation or desumoylation can lead to disturbances in the cell's genetic integrity, which in turn may contribute to carcinogenesis (Pagano and Benmaamar 2003). Sumoylation is involved in for instance cancer metastasis (Kim and Baek 2006) and some types of inflammations are associated with altered activity of sumoylation (Liu and Shuai 2008). Detailed roles of sumoylation in the origin of tumors are not well known, but sumoylation of receptor-mediated signaling mechanisms is strongly involved. Nuclear receptors are ligand-dependent transcription factors that regulate cell differentiation and cell growth in several different types of cells, and during the development of cancer (Wu and Mo 2007).

Sumoylation regulates the activity of a wide variety of proteins including p53, retinoblastoma protein (pRB) and Mdm2, all of which have important roles in for instance regulating the proceeding of the cell cycle (Sarge and Park-Sarge 2009). Sumoylation has been shown for instance to affect the stability and function of p53 on K386 (Gostissa et al. 1999; Kim and Baek 2006). Several proteins participating in the sumoylation conjugation pathway have been studied in the context of cancer research. Ubc9 in particular has received a lot of attention in cancer research regarding sumoylation. Unusually high amounts of Ubc9 have been detected in for example lung adenocarcinoma and melanoma (Hoeller and Dikic 2009). Because Ubc9 is the only known protein to be able to conjugate SUMO to its target protein it is an appealing target for future medication and treatment based on altering SUMO conjugation (Moschos and Mo 2006). Potential ways to inhibit Ubc9 include affecting the association between E1 and Ubc9, blocking the active site of Ubc9 and blocking the binding to target proteins (Hoeller and Dikic 2009).

The different SUMO E3 ligases that enhance the sumoylation efficiency of disease-associated SUMO substrates are also targets in the development of new treatments in SUMO-associated diseases. This is mainly because of remarkably increased selectivity among E3 ligases compared to more general attempts in trying to regulate the activity of the only so far identified conjugating protein Ubc9 (Sarge and Park-Sarge 2009). In 2004 it was reported that the E3 ligase PIAS3 expression was increased in 100 out of 103 samples that contained cancer tumors from for instance prostate, brain, breast and lung (Wang and Banerjee 2004).

The complex role of sumoylation in the development of cancer arises since it has also been reported that the desumoylating protein SENP1 occurs in higher amounts in prostate cancer. Overexpression of SENP1 in transgenic mice leads to tumorigenesis (Cheng et al. 2006) and increased expression of SENP1 have also been reported to exist in thyroid cancer (Jacques et al. 2005).

Sumoylation maintains brain functions and thus sumoylation can affect neurodegenerative processes (Bonifati et al. 2004). Several SUMO target proteins have been associated with several neurodegenerative diseases. Among these proteins are amyloid precursor protein (APP) which is associated with Alzheimer's disease, Huntingtin which is associated with Huntington's disease, and Tau which is associated with both Parkinson's and Alzheimer's diseases (Dorval and Fraser 2007).

During infection, some pathogenic bacteria can manipulate the host cell's PTMs. In this way the bacteria can avoid the host's immune response and potentiate its own replication (Rytkönen and Holden 2007). Among these modifications are phosphorylation and ubiquitination, but also sumoylation whose role in this context is less known (Ribet et al. 2010). In terms of immunity, SUMO is not only involved in the development of autoimmune diseases, but the role of sumoylation in the immune response is also important as pathogens during bacterial and viral infections seek to overcome the host defense by inhibiting sumoylation in host cells. This is because sumoylation regulates NF-κB and IFNs pathways which are major pathways within the innate immunity (Adorisio et al. 2017). Infection in eukaryotes by *Listeria monocytogenes* leads to proteasome-independent degradation of Ubc9 mediated by the bacterial virulence factor listeriolysin O (LLO). This in turn reduces the amount of SUMO conjugated proteins. The efficiency of the bacterial

infection is dampened by increased presence of SUMO, which indicates that LLO dampens the host cell's immune response by decreasing the sumoylation efficiency (Ribet et al. 2010). Boggio et al. showed in 2004 that the adenoviral protein Gam1 inactivates SAE1/SAE2 *in vivo*. The inactivation results in the halt of the sumoylation conjugation pathway (Boggio et al. 2004).

Recent research has uncovered cardiac function to be included among the physiological environments in which SUMO operates. The promyelocytic leukemia protein (PML) is sumoylated and ubiquitinated upon exposure to arsenic trioxide. PML/RNF4 (a SUMO-targeted ubiquitin ligase) mediates important functions in cardiac fibrosis, which in turn may be plausible therapeutic targets for cardiac fibrosis and heart failure treatment by interfering/steering this pathway (Figure 20) (Yang et al. 2017).

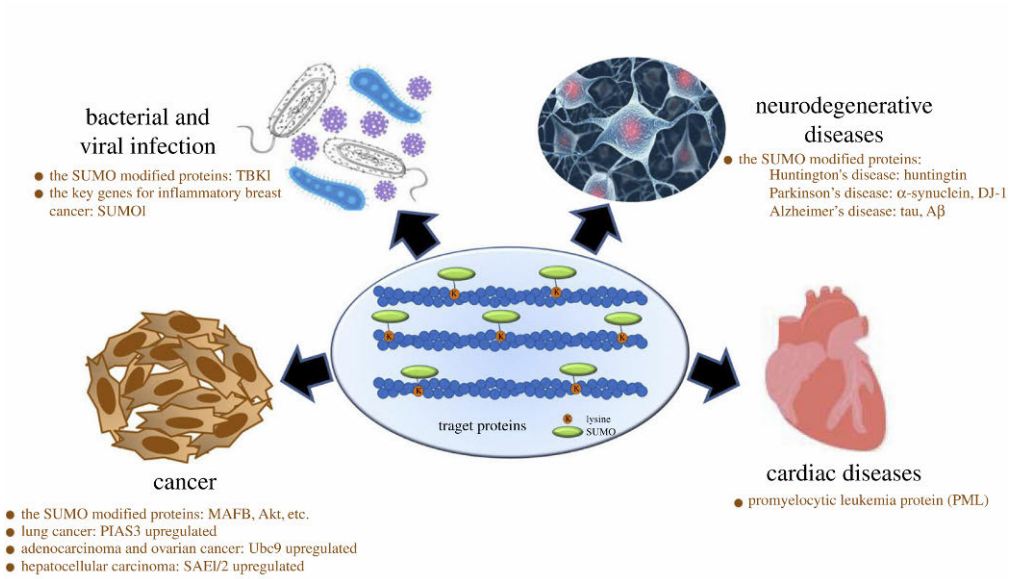

Figure 20. The relationship between SUMO-conjugated proteins and different diseases. Some of the SUMO target proteins, and member proteins of the SUMO conjugation machinery are displayed. The figure is adapted from (Yang et al. 2017).

3. KERATINS AS REGULATORS OF INTESTINAL HOMEOSTASIS

3.1 The colon and its anatomy

Organs of the gastrointestinal tract including the esophagus, stomach, small intestine and the large intestine/colon act in symbiosis with the liver, pancreas and gall bladder to maintain the vital undertakings of digestion and absorption (Thompson et al. 2018). Epithelial tissue borders the outer surfaces of blood vessels and organs, and also the inner surfaces of cavities in several internal organs. The epithelium of the gastrointestinal tract is constantly self-renewing and is also the fastest self-renewing tissue in mammals (Schepers and Clevers 2012). The gastrointestinal tract comprises the upper and lower gastrointestinal tract out of which the latter is organized into the small intestine (duodenum/jejunum, and ileum) and the colon/large intestine (Thompson et al. 2018). The colon is divided into proximal colon (cecum, ascending colon, hepatic flexure, transverse colon) and the distal colon (descending colon, sigmoid colon, rectum) (Lin et al. 2016).

The intestinal epithelial tissue is composed of an expansive surface area suitable for uptake of nutrients. The intestinal epithelium also mediates immune homeostasis and sustains a neighboring barrier to the external surroundings (Smith et al. 2016). In the intestine, it is specifically the intestinal epithelium that has a major role in immune homeostasis. This occurs not only by the epithelium physically being a barricade against extrinsic antigens and commensal bacteria thus segregating the host tissue from the unwanted pathogens, but also by the epithelium for instance directly processing antigens (Park et al. 2018). The primary function of the epithelium of both the small intestine and the colon is absorption. The epithelial cells within the colon mainly absorb water and electrolytes. The small intestine and the colon have many common characteristic features, but perhaps the most distinguishing feature is that unlike the small intestine, the colon lacks villi and Paneth cells. However, occasionally Paneth cells can be abnormally present in the colon as well in cases of inflammatory bowel disease (IBD) and colorectal cancer, emphasizing the importance of comprehension of the mechanisms related to the colon (Thompson et al. 2018).

3.1.1 Proliferation and differentiation of colonic epithelial stem cells and progenitor cells

Many different tissue types including skin, stomach and intestinal tissue are in a state of constant and permanent regeneration. Newer cells arising from stem cells are non-stop replacing older cells within the tissue (Clevers et al. 2014). The intestine's epithelial lining is continuously coming into contact with microbes, gastric acids and malicious consumed substances creating a hostile surrounding (van Rheenen and Bruens 2017). Continual mechanical and chemical stress in the form of accumulation of potential physical injuries forces the epithelial layer to set up a protective mechanism in the form of regenerating itself (Smith et al. 2016) and therefore the intestinal epithelial tissue is constantly regenerated fast, every 4-5 days (Naito et al. 2017) making the intestinal epithelium one of the favorite tissues to study the maintenance and differentiation of stem cells (Park et al. 2018). Epithelial cells are one of the most rapidly renewing cells in mammalians and are thus

particularly vulnerable to enter malignant transformation (Pastuła and Marcinkiewicz 2018). The molecular mechanisms that steer the regulation of the proceedings of tissue renewal are malfunctioned in settings such as IBD, which consequently increases the risk of colon cancer development (Reynolds et al. 2014).

In adult organisms it is specifically epithelial stem cells that regulate epithelial tissue renewal for instance in the gastrointestinal tract, respiratory tract and skin (Pastuła and Marcinkiewicz 2018). The colonic epithelium is composed of millions of invaginations called crypts, and every crypt is the self-renewing unit of the tissue (Reynolds et al. 2014). Colonic epithelial cells are developed from progenitor cells in the proliferative crypts (Schepers and Clevers 2012). There are 2000-3000 cells totally within a proliferative crypt (Vinson et al. 2016). At the bottom/base of the crypts lie intestinal stem cells (ISCs) which proliferate approximately once a day (Figure 21) (Schepers and Clevers 2012).

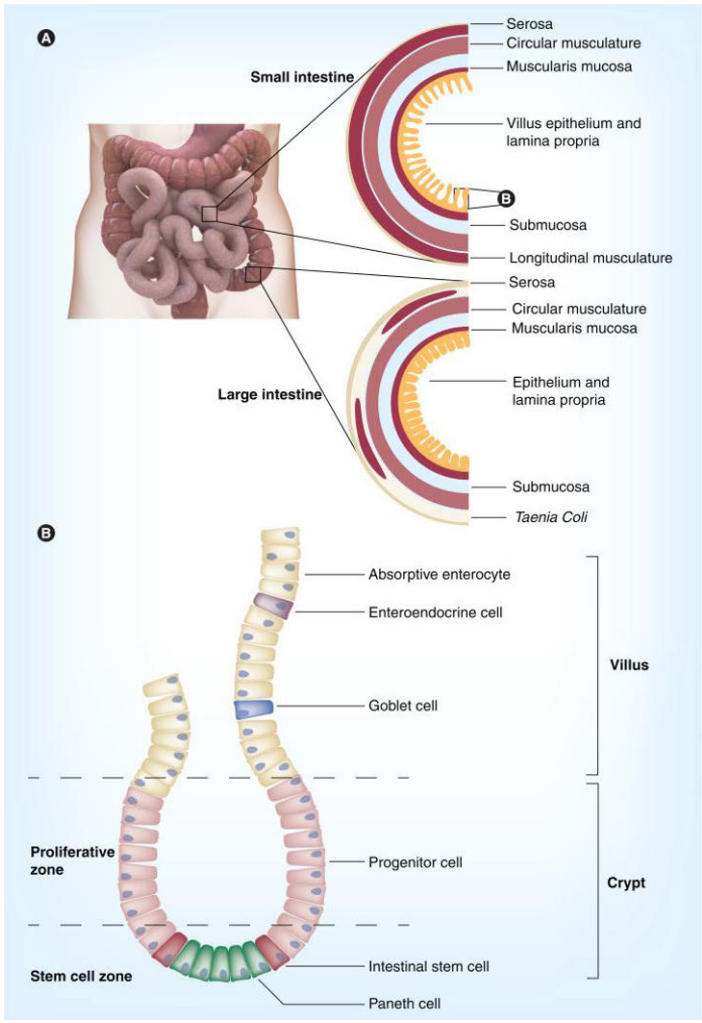

Figure 21. Histology of intestinal layers and crypts (A) In cross-section, both the small intestine and the colon comprise outer layers of serosa and both longitudinal and circular musculature. The colon has large muscular ribbons called taenia coli which aid in contraction and peristalsis. The middle layers consist of submucosa and muscularis mucosa. The innermost layers are called the lamina propria and epithelium. **(B)** The crypt and the villus are the self-renewing units of the tissue in the mammalian intestine. Intestinal crypts contain resident stem cells capable of generating all cell types of the mature epithelium. Enterocytes, goblet cells and enteroendocrine cells are all already differentiated cells. Note that the colon/large intestine that follows the small intestine does not contain villi or Paneth cells. The figure is adapted from (Howell and Wells 2011).

These intestinal stem cells consist of crypt base columnar (CBC) cells which can be identified by ISC marker genes including *LGR5*, *OLFM4*, *SMOC2* and *SLC12A2*. The other group of ISCs consists of +4 stem cells located at the '+4' region of the crypts which preferentially express genes including *BMII* and *HopX* (Suzuki et al. 2018).

Epithelial stem cells at the crypt bottoms can proliferate either into two daughter stem cells (symmetric proliferation) accounting for 5% of the mentioned proliferations, or into one stem cell and one TA progenitor cell (asymmetric proliferation) accounting for 95% of the mentioned proliferations (van der Flier and Clevers 2009). The colonic TA cells proliferate once every 12-16 hours, undergo 4-5 proliferations and migrate towards the top of the crypts after which the colonic TA cells differentiate into some of the cell types within the absorptive or secretory cell lineages in the colon (Figure 22). In the small intestine, the TA cells can differentiate also into Paneth cells which secrete bactericidal products such as lysozymes and defensins (Cui and Chang 2016). The amount of these different differentiated cell types in relation to each other varies according to the location in the crypt (Weichselbaum and Klein 2018).

The absorptive cell lineage consists only of enterocytes that absorb nutrients such as water and ions (Tetteh et al. 2016). Enterocytes make up over 80% of all intestinal epithelial cells (van der Flier and Clevers 2009). The secretory cell lineage in the colon comprises goblet cells and enteroendocrine cells. In the small intestine, also Paneth cells are a member of the different cell types in the secretory cell fate. Goblet cells make up only about 16% of the cells in the descending colon (van der Flier and Clevers 2009) and secrete protective mucins (mixture of inorganic salts, lysozymes, immunoglobulins, and glycoproteins such as lactoferrin) (Sancho et al. 2015). The epithelial cells are covered by a mucus layer which consists mainly of the gel-forming mucins which are part of the innate immunity (Johansson et al. 2008). The mucus barrier protects the colon from mechanical stress, gastric acids, and disease-causing pathogens such as bacteria. The mucus also protects the crypt stem cells (Vinson et al. 2016) from acquiring mutations by preventing toxins especially in the form of carcinogens from gaining access to the crypt (Garg et al. 2007).

The protective role of the mucus is evident for instance from the fact that mice lacking the protein Mucin 2 (Muc2) spontaneously develop colitis (Van der Sluis et al. 2006). Most goblet cells also secrete trefoil factors (TFFs) which also contribute to the preservation of the integrity of the mucus barrier (Thim et al. 2002). Enteroendocrine cells constitute only 1% of the cells in colonic crypts and secrete several different hormones such as serotonin, secretin and somatostatin (Sancho et al. 2015) which have functions in the regulation of the colon's motility and peristalsis which in turn refers to the wave-like contraction of the digestive tract that make food pass it (Gunawardene et al. 2011).

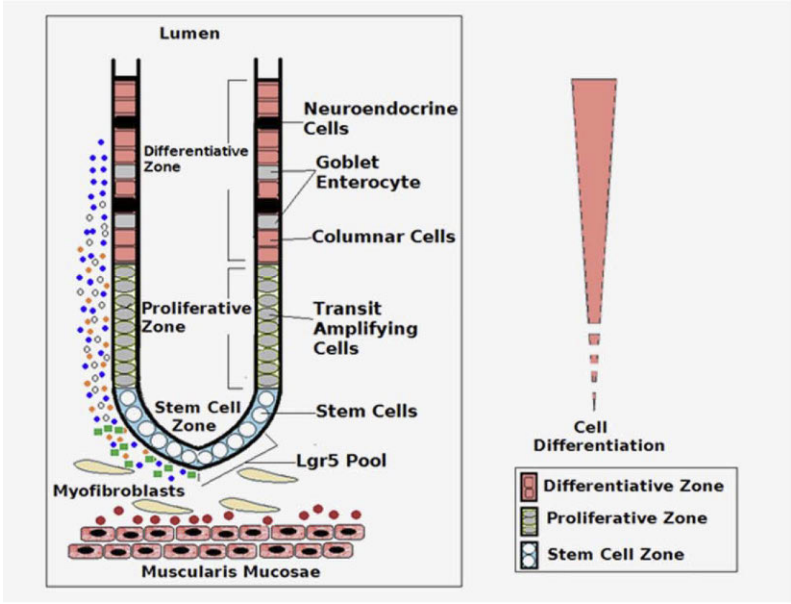

Figure 22. Stem cell differentiation in the human colon crypt, and the different components in the different zones. The figure is modified from (Vinson et al. 2016).

Math1/Atoh1 is considered to be the gatekeeper of TA cells entering the secretory cell lineage through differentiation (Clevers 2013) whereas Neurogenin3 is needed for the gastric TA cells to be able to differentiate specifically into enteroendocrine cells within the secretory cell fate (Lee et al. 2002). The formation of intestinal tuft cells which represent a very rare cell type is not dependent on the actions of Math1/Atoh1 and tuft cells are therefore not in most cases considered to be secretory cells. The function of the tuft cells is less known (Sancho et al. 2015), but it has been shown that tuft cells are located close to the colonic stem cell zone in the crypt and scattered along the crypt axis (in the small intestine along the crypt-villus axis). Various data also indicate that tuft cells are part of a chemosensory system which consists of chemosensory cells along the gastrointestinal and respiratory tract (Middelhoff et al. 2017). The life cycle of an intestinal epithelial cell under normal conditions *in vivo* is terminated by apoptosis 4-5 days post formation (Clevers 2013).

3.2 Keratin intermediate filaments

The cellular cytoskeleton is composed of intermediate filaments (IFs), actin filaments and microtubules and its most essential tasks are to support the composition and motility of cells (Fletcher and Mullins 2010). IF proteins are the key components of the cytoskeleton and the nuclear membrane in most eukaryotic cells (Liu et al. 2017). IFs are a larger group of proteins that have common structural characteristics and they also form nonpolar fibrous filaments with a length of approximately 10 nM, which is “intermediate” in relation to the polar filaments formed by actin microfilaments (6-8 nm) and microtubules (25 nm) (Cruz et al. 2018). Despite the fact that IFs belong to one of the three essential cytoskeletal groups in vertebrates they are still the least comprehended regarding both function and structure.

The complexity to understand IFs from different aspects arises partly from a vast amount of genes encoding them in a cell- and tissue-specific manner (Goldman et al. 2012). Different IFs are therefore synthesized in different cell types (Figure 23) (Eriksson et al. 2009).

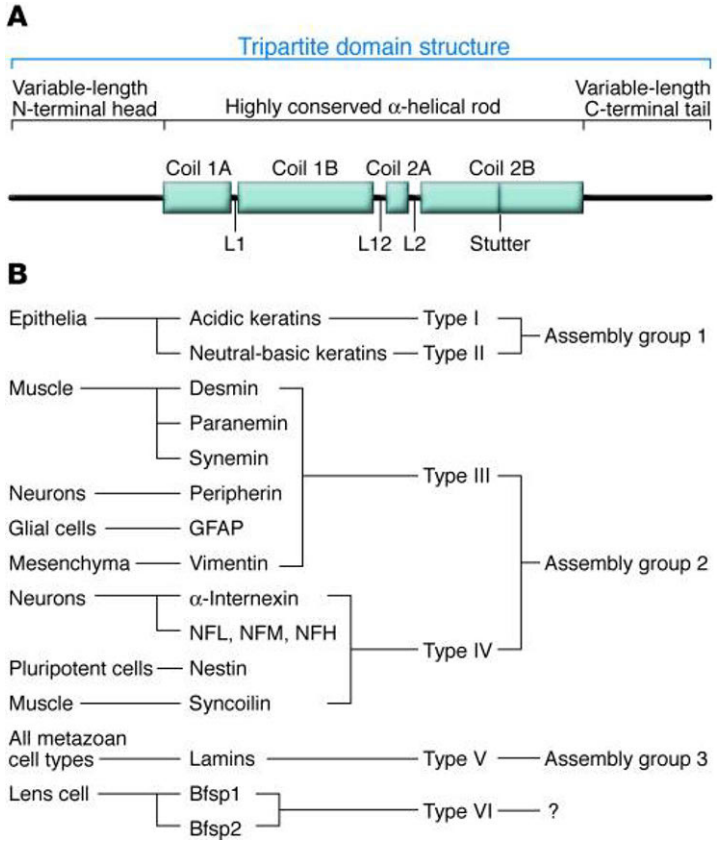

Figure 23. Overview of the IF protein family. A) All IFs have a conserved central α-helical rod domain which is bordered by a non–α-helical tail and head domains. **B)** The IFs are grouped into distinct classes as indicated. The grouping is based on rod domain amino acid sequence, net acidic charge and secondary structure predictions. The groups are subdivided into assembly groups based on co-polymerization ability. NFL, NFM and NFH stand for low-, middle- and high- molecular weight neurofilament subunits. The figure is adapted from (Eriksson et al. 2009).

3.2.1 The keratin family

Keratin proteins are the largest subfamily of intermediate filament proteins of the cytoskeleton and are primarily expressed in the cytoplasm of epithelial cells (Cruz et al. 2018). Keratins form the cytoskeleton of all epithelial cells (Loschke et al. 2015). Keratin assemblies have a dynamic nature both as only individual 10 nm filaments and as more advanced filament networks (Jacob et al. 2018).

Keratin monomers are highly unstable whereas filaments formed by keratins are very stable (Jacob et al. 2018). Unlike other IFs, keratin assemblies are initiated when a type I keratin monomer (keratins 9-20) forms a heterodimer with a type II keratin monomer (keratins 1-8) by obligate non-covalent heteropolymerization (Moll et al. 1982). These heterodimers form tetramers which can further form larger assemblies, filaments (Haines and Lane 2012) whose main function is to provide epithelial tissue integrity during mechanical force and non-mechanical stresses (Loschke et al. 2015).

Keratins are divided into two subgroups based on protein sequence: acidic type I keratins and neutral/basic type II keratins (Jacob et al. 2018). The structure of keratins resemble that of all other IFs and comprises a central coil-coil α-helical ~310 amino acid long rod domain which is bordered by non–α-helical tail and head domains which have various lengths and sequence composition (Figure 24) (Loschke et al. 2015).

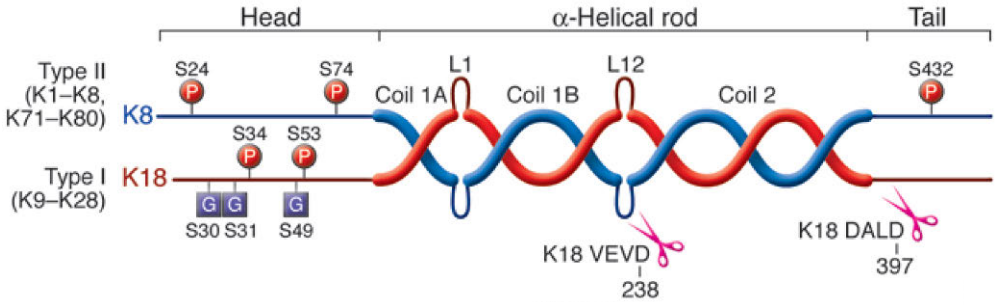

Figure 24. The keratin prototype structure. Keratins form noncovalent heterodimers and have a structure that is similar to all other IFs. The keratin protein structure comprises a central coil-coil α-helical rod domain which is bordered by non–α-helical tail and head domains. These tail and head domains are particularly interesting since most PTMs (P=phosphorylation, and G=glycosylation) on keratins occur on these domains. The rod domain in the middle is divided into subdomains (coils) and linkers (L) as indicated. The indicated caspase cleavage site VEVD on K18 is conserved in IF proteins whereas the indicated site DALD on K18 is unique to K18. The figure is modified from (Omary et al. 2009).

Keratins are encoded by evolutionarily conserved genes: type I keratins by 28 genes and type II keratins by 26 genes, which comprises a major portion of the 70 genes encoding for all IFs (Jacob et al. 2018). IFs are expressed in a cell-specific manner. For instance, keratins are mainly expressed in epithelial cells, vimentin in mesenchymal cells, glial fibrillary acidic protein (GFAP) in glial and stellate cells, and desmin in myocytes (Omary 2017). The expression of keratin-encoding genes is regulated in a **tissue**- (e.g. simple or stratified, hard or soft), **differentiation**- (less differentiated basal or more differentiated suprabasal cells) and **context-dependent** type (normal homestoasis or tissue repair/disease) manner (Jacob et al. 2018). The vast complexity and diversity observed in the expression of keratins creates challenges in the comprehension of the cell biological roles of keratins. For example, K7 is expressed mostly in the lower parts of the crypts whereas K20 is being expressed in the differentiated cell population in the top parts of the crypt. An example from within one cell type is for instance murine pancreas acinar cells that have K8 and K18 filaments throughout the cytoplasm whereas K19 is in basal conditions expressed only at the apico-lateral cell membrane (Strnad et al. 2016). This also highlights the essential feature of keratins that specific keratin pairs predominate over single keratin isoforms (Omary et al. 2009).

Keratins can be classified into three functional groups: 1) **simple epithelial keratins** (SEKs) expressed in single-layered epithelia such as the intestine, liver and glandular secretory cells 2) **barrier keratins** expressed in stratified (contains two or more layers of

cells) epithelia including skin epidermis 3) the **harder structural keratins** forming hair and nail (Table 3) (Haines and Lane 2012).

	Keratin	Epithelial tissue	Polymerisation partner in vivo
Type I			
Simple	K18	Simple epithelia (e.g. liver, pancreas, colon, lung)	K8, K7
	K20	Simple epithelia, especially gastrointestinal	K8, (K7)
Barrier	K9	Stratified cornifying epithelia; palm, sole	(K1)
	K10	Stratified cornifying epithelia, suprabasal	K1
	K12	Stratified epithelia; cornea	K3
	K13	Stratified epithelia, non-cornifying; suprabasal	K4
	K14	Stratified and complex epithelia; basal	K5
	K15	Stratified epithelia	(K5)
	K16	Stratified epithelia; induced during stress, fast turnover; suprabasal	K6a
	K17	Stratified epithelia; induced during stress, fast turnover	K6b
	K19	Simple and stratified epithelia	K8
	K23, K24	Epithelia	
Structural	K25, K26, K27, K28	Stratified epithelia: hair follicle sheath	
	K31, K32, K33a, K33b, K34, K35, K36, K37, K38, K39, K40	Stratified epithelia: hair, hard structures	
Type II			
Simple	K7, K8	Simple epithelia	K18
Barrier	K1	Stratified epithelia, cornifying, suprabasal	K10
	K2	Stratified cornifying epithelia, late suprabasal	(K10)
	K3	Stratified epithelia, cornea	K12
	K4	Stratified epithelia, non-cornifying; suprabasal	K13
	K5	Stratified and complex epithelia; basal cells	K14, (K15)
	K6a	Stratified epithelia; induced during stress, fast turnover	K16
	K6b	Stratified epithelia; induced during stress, fast turnover	K17
	K6c	Epithelia	
	K76	Stratified cornifying epithelia, oral, suprabasal	(K10)
	K78, K79, K80	Epithelia	
Structural	K75	Stratified epithelia: hair follicle	
	K71, K72, K73, K74	Stratified epithelia: hair follicle sheath	
	K81, K82, K83, K84, K85, K86	Stratified epithelia: hair, hard structures	

Table 3. Expression of keratin proteins in epithelial tissues. The figure is adapted from (Haines and Lane 2012).

Simple epithelial keratins have a quite slow turnover with a half-life of 3.5 days (84 hours) in mouse hepatocytes, a form of liver epithelial cell (Denk et al. 1987). K8 belonging to SEKs is along with K18 the main component, not only of all keratin isoforms, but also of the entire intermediate filament cytoskeleton (Sun et al. 2013). K8 is therefore also the main intermediate filament protein of simple or single layered epithelia (Liu et al. 2017). K8 and K18 are complemented in most epithelia by K7, K19, K20, K23 and K80 (Loschke et al. 2015). K8 belongs to the type II keratins, pairs predominantly with the type I K18, and is mainly expressed in the epithelia of the intestine, liver, exocrine pancreas and mammary glands (Sun et al. 2013; Bozza et al. 2018), but K8 is also expressed in small amounts in stratified epithelia in the skin, and in non-epithelial tissues such as the heart muscle. K8/K18 is localized preferentially in the cytoplasm (Omary et al. 2009). For instance, K8 and K18 are the only keratin isoforms that adult liver hepatocytes express (Jacob et al. 2018). SEKs (K8 and K18 in particular) differ from other keratins in several ways. For instance, SEK human mutations lack the ends of the rod domain, SEKs are more soluble than other keratins, and also the expression levels of SEKs are lower than those of epidermal keratins. The epidermis is the outermost of the three layers that the skin consists of, the two inner layers are called the dermis and hypodermis (Omary et al. 2009). In stratified epithelial tissue the most abundant keratin isoforms are K5, K14, K6 and K16 (Moll et al. 1982). Keratins are also the main proteins of the epidermis accounting for 17-27% of all proteins in basal, and 50% in terminally differentiated keratinocytes which are the predominant cell types in the epidermis (Loschke et al. 2015).

3.3 Keratins in the colon

In the beginning of this millennium the knowledge of the functions of keratins specifically in the intestine and their role in disease development was based not on K8 mutations, but predominantly on the K8-null mouse phenotype which had indicated that intestinal keratins regulate differentiation, cell growth, and targeting of proteins to the apical compartment (Majumdar et al. 2012).

K8, K18 and K19 constitute the largest group of the keratin isoforms being expressed in the intestine (Moll et al. 1982) and **these proteins are distributed mostly evenly throughout the entire colonic epithelial crypts.** K18 is expressed abundantly particularly in goblet cells (Zhou et al. 2003). K20 is found in the differentiated enteroendocrine cells of the most upper areas of the crypts whereas K7 is expressed at low levels mostly at the crypt base in the proliferating cell compartment. Additionally, K7 can be expressed in goblet cells in the upper parts of the crypts. Also K23 has been observed in the human colon, but only in human colon cancers, not in healthy colonic tissue (Omary et al. 2009).

K8 and K18 are the first IF proteins being expressed during mouse embryogenesis. The C57B1/6 $K8^{-/-}$ mouse strain which was generated in the beginning of the 1990's, suffers with a 94% rate from embryonic lethality in no more than 12-13 days from initiation of embryogenesis, while the remaining small population of 6% develop into adulthood (Baribault et al. 1993). Despite the heterodimerization of K8 and K18, the mouse strain C57B1/7 $K18^{-/-}$ does not undergo embryonic lethality, but is instead viable and displays a normal life span (Magin et al. 1998). Another transgenic mouse model, FVB/N $K8^{-/-}$ allows for 55% of mice embryos to escape embryonic lethality, although they develop colorectal hyperplasia and colonic inflammation showing that genetic modifiers of K8 affect not only embryogenesis, but also adult tissues (Baribault et al. 1994). $K8^{-/-}$ in the colon diminishes the levels of the other SEKs K7 and K18-K20 within the colon (Asghar et al. 2015). K8-null mice have been shown to suffer from a vast amount of different medical conditions including diarrhea (Baribault et al. 1994; Toivola et al. 2004), abnormal colonic ion transport (Toivola et al. 2004), chronic spontaneous T-helper cell (Th2) colitis (Habtezion et al. 2005), crypt hyperproliferation, increased resistance to colonic apoptosis (Habtezion et al. 2011) and colonic homeostasis disruption (Helenius et al. 2015). K8-null mice have also been shown to generate increased amount of goblet cells and enteroendocrine cells at the expense of the amount of enterocytes, steering the final cell lineage and cell type of differentiation of TA cells (Lähdeniemi et al. 2017).

K8 expression levels matter in the development of cancer within the colon and K8 is downregulated in humans who have developed colon cancer. By carrying out a protective role on the permeability of the colon, higher amounts of K8 consequently prevent mice from developing colitis and colitis-associated colorectal cancer. In addition to modifying the permeability of the colon, K8 also modifies the gut microbiome composition (Liu et al. 2017). Additionally, K8-null mice do not develop colonic tumors spontaneously although they are predisposed to developing azoxymethane (a colon-specific carcinogen of high stability)-induced colorectal cancer (Misiorek et al. 2016). Keratin filament assembly-distracting mutations have been observed in IBD patients (Owens et al. 2004), and decreased levels of K8, K18 and K19 in colonic acute inflammation (Corfe et al. 2015). Also links between disease and the phosphorylation status of K8 have been discovered.

Loss of K8 phosphorylation has been detected in the colorectal carcinoma cell line HCT116 (Khapare et al. 2012) and reduced K8 phosphorylation in colonic acute inflammation (Corfe et al. 2015). Interleukin 6 (IL-6) in turn has been shown to induce K8 phosphorylation, and also to enhance K8 and K18 expression, which seems to prevent development of intestinal disorders such as IBD whose inflammatory response is a result of weakened intestinal barrier and its related dysfunction (Wang et al. 2007c).

Like keratins in other cellular contexts, also intestinal keratins provide tissue integrity, and they are also upregulated, reorganized and post-translationally modified during cellular stress and recovery (Haines and Lane 2012). For details regarding the role of keratins in battling against cell stress, please see section "3.4.2 Keratins as cellular stress protectors".

3.4 Keratins in biological functions

Keratins are members of a protein family that is imperative for the formation of for instance hair. Keratins also protect epithelial cells from different forms of damage, for example by being abundant in the epidermis (Liu et al. 2018). In surface-exposed stratified epithelia high amounts of cytoplasmic keratin filaments provide structural support by forming a cytoplasmic network with attachments at the surface of the nucleus, and at adhesive structures (e.g. cell-matrix or cell-cell) that cross the cell membrane (Jacob et al. 2018).

In simple epithelial tissues the main roles of keratins are to protect the cell against chemical stresses and contribute to the preservation of cell polarity. Keratins also participate in epithelial tissue growth by stimulating the cell cycle and protein synthesis, and by activating immune or inflammatory mediators that stimulate epithelial tissue growth (Jacob et al. 2018). Keratin filaments also interact with a vast amount of proteins in order to function as scaffolds to organize signaling platforms (Cruz et al. 2018), and modify protein localization and targeting. These keratin-interacting proteins include among others hsp70, kinases and phosphatases, 14-3-3 proteins and Fas receptors and serve to aid in accurate function and localization of organelles including Golgi and mitochondria. Keratins also have crucial context-dependet roles in whether and when cells should undergo apoptosis (Figure 25) (Omary et al. 2009). Keratin-null (K8/K18) hepatocytes are highly more susceptible to undergo apoptosis compared to their wild-type counterparts. However, K8-null enterocytes are nonetheless more resistant to apoptosis compared to wild-type enterocytes (Omary 2017). In epidermal cells keratin filaments bind to desmoplakin and hence link into desmosomes forming specific cytoskeletal structures which connect neighboring cells (Kouklis et al. 1994).

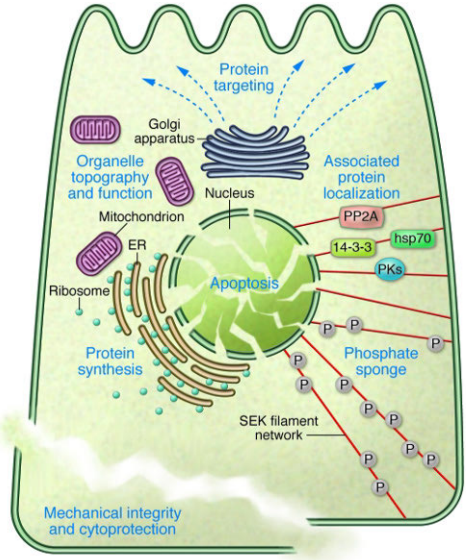

Figure 25. Overview of the functions of simple epithelial keratins. Essential roles of SEKs include participation in the maintenance of mechanical integrity and cytoprotection from apoptosis, and serving as phosphate sponges upon cell stress (Omary et al. 2009).

3.4.1 Keratins as cellular stress protectors

IFs regulate a broad spectrum of differentiation and proliferation events and in addition to keratins also other IF proteins such as nestin (Sahlgren et al. 2003) have been shown to have important roles in tissue development. During early embryonic development, the expression of type II keratins precedes that of type I keratins (Lu et al. 2005), but both keratin types are required to form regular keratin filaments in the cytoplasm (Omary et al. 2009). K8 and K18 are not only the most abundant keratin isoforms in epithelial cells, but they also constitute the most common keratin proteins being synthesized early in development (Strnad et al. 2016).

Adult hepatocytes do not express K7, K14 or K19, but embryonic hepatoblasts express all of these keratin isoforms (Omary et al. 2009). Keratins and vimentin are co-expressed in fetal kidney, but in contrast, they are almost never present in the same cell in adults (Moll et al. 1991). In the beginning of this millennium the knowledge of keratins in embryonic development took several steps forward. It was shown that mouse embryonic lethality occurs if K8, K18 and K19 are absent from the placenta where the mentioned keratin isoforms are expressed in normal conditions co-operating to secure healthy development of the placental tissue. Data has shown that targeted deletion of K18 and K19 results in cytolysis (the rupture of cell membranes and loss of cytoplasm) in trophoblast giant cells (cells forming the outer layer of blastocysts which are structures that form the embryo) causing early embryonic lethality at around E9.5 (Hesse et al. 2000). Trophoblast giant cell layer failure is the predominant defect causing also $K8^{-/-}$ embryonic lethality (Tamai et al. 2000; Jaquemar et al. 2003). Another newer study states that mice lacking the whole type II gene cluster suffers from embryonic lethality at E9.5, but not due to embryonic epithelial cytolysis, but rather due to the mislocalization of the glucose transporters GLUT1 and GLUT3, increased AMP kinase activity, reduced mTORC1 activity and finally repressed protein biosynthesis (Vijayaraj et al. 2009). Directly adjacent to the placenta in the decidual tissue, absence of the entire type II keratin gene cluster also generates hyperoxia (exposure of tissues to abnormally high levels of oxygen) further resulting in deregulated

vasculogenesis through malfunctioned HIF-1α and defective vascular endothelial growth factor signaling (Kröger et al. 2011).

Keratins also have a role in the migration of cells during embryogenesis. Mechanical force (in the form of tension on cadherin adhesions) distributed to C-cadherins (a type of cell adhesion molecule) on mesendoderm cells (an embryonic tissue layer which differentiates into mesoderm and endoderm) of *Xenopus* reorganizes keratin filaments towards these mechanically stressed sites. The cadherin-keratin complex then directs collective migration of individual cells in a migrating tissue (Weber et al. 2012).

Accurately timed assembly and disassembly of keratin filaments are needed during alteration of keratin expression patterns during cell differentiation and cell stress. The transition from assembly to disassembly or the contrary is not full and complete, but gradual in order to sustain a constant keratin filament network providing structural integrity for the cell at all times. Keratin filament assembly/disassembly alteration takes place for instance in the epidermis where K5 and K14 are the main keratin isoforms in basal/proliferative cells whereas K1 and K10 are being expressed in suprabasal cells (already sealed for differentiation) (Haines and Lane 2012). Furthermore, $K9^{-/-}$ mice display impaired differentiation and impaired structural integrity in the epidermis (Fu et al. 2014). Relatively recently also K24 has been discovered to be required for epidermal differentiation upon CaCl2-induced differentiation (Min et al. 2017). Thus, alterations in the kinetics of keratin filament assembly/disassembly can interrupt essential cell functions such as differentiation (Haines and Lane 2012). The importance of correctly timed keratin filament network assembly/disassembly is found for instance in the skin disease Dowling-Meara epidermolysis bullosa simplex (EBS) which is caused by excessive buildup of keratin clusters and is characterized by excessive skin blisters (Ishida-Yamamoto et al. 1991).

3.4.2 Keratins as cellular stress protectors

Different forms of stress conditions are constantly being targeted against cells. These stresses harming vital parts of the cell including DNA and proteins are either of extrinsic origin such as heat and toxins, or of intrinsic origin such as genetic or ER stress (Figure 26) (Toivola et al. 2010).

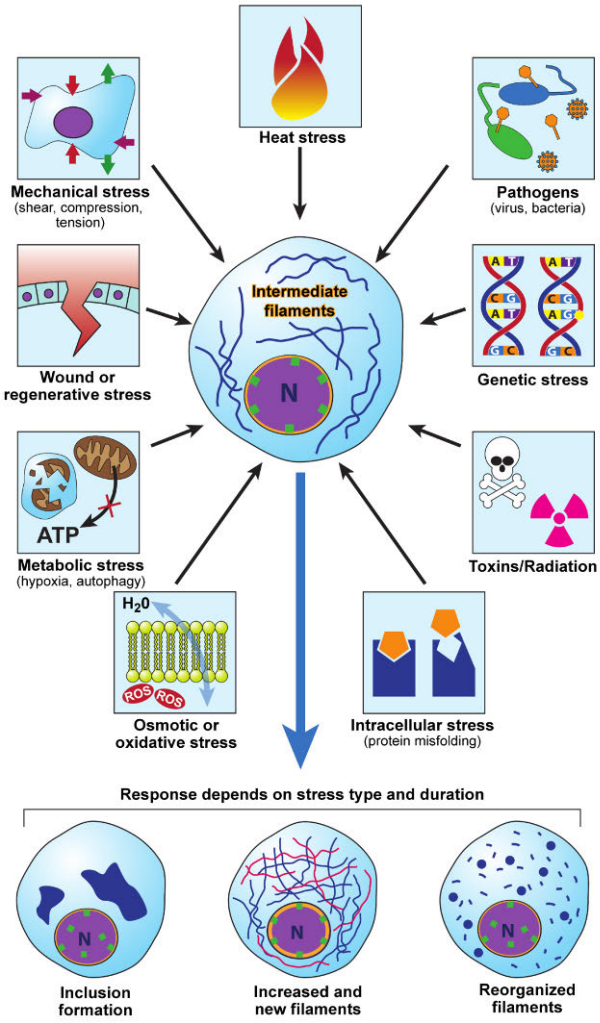

Figure 26. Different stress conditions that modify intermediate filaments. Cells are being exposed to several different forms of cellular stresses that stem from the intrinsic or extrinsic environment. The behavior by how intermediate filaments respond to a specific stress is influenced by the type and duration of the stress. The figure is adapted from (Toivola et al., 2010).

As a consequence of cell stress the expression of several keratin isoforms is upregulated, especially regarding K6a, K6b, K16 and K17 whose expression is enhanced by oxidative and UV stress, wounds and inflammatory cytokines (Haines and Lane 2012).

Approximately 5% of keratins exist in a soluble state at any given time point and the rest of keratin proteins exist in an insoluble filamentous state. The state is heavily influenced by close environmental circumstances, particularly cellular stress and PTMs (Majumdar et al. 2012). For instance, the dynamic filament networks formed by keratins provide structural integrity of epithelia by resilience against mechanical cell stress making keratins essential for epithelial stress protection (Figure 27). Keratin filaments are flexible, tough and able to withstand substantial mechanical powers especially in stratified epithelia including the epidermis, by diminishing the mechanical stress from one particular spot into a larger epithelial tissue area (Haines and Lane 2012). Wildtype keratin filaments are able

to resist mechanical stress in the form of repeated stretch much more efficiently than keratin filaments with specific K5/K14 mutations that cause EBS (Russell et al. 2004).

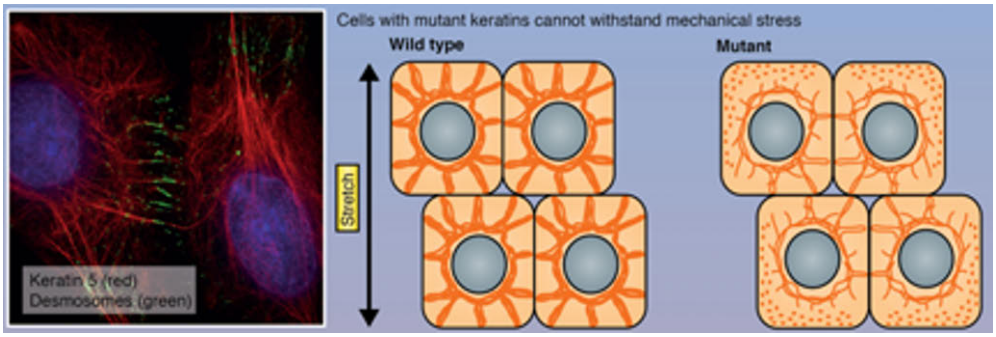

Figure 27. Keratins provide cells with structural integrity by mechanical resilience against cellular stress conditions. The figure is modified from (Haines and Lane 2012).

Both keratin dimers and tetramers can be formed even in a strongly denaturing environment, which emphasizes the resilience and strength of type I and II keratins (Jacob et al. 2018). Keratin filaments are capable of remodeling and/or undergoing fast turnover as a reaction to not only different forms of cellular stresses, but also mitosis and cell migration (Jacob et al. 2018). These capabilities arise from keratins associating during cellular stress, mitosis and migration with various other proteins and keratins undergoing distinct PTMs, especially phosphorylation, but also sumoylation, acetylation, glucosylation, prenylation, transamidation and caspase cleavage (Snider and Omary 2014).

Phosphorylation of K8 protects, but does not prevent it from undergoing ubiquitin-mediated degradation (Ku and Omary 2000). K8/K18 is hyperphosphorylated in Mallory bodies (MBs) (Zatloukal et al. 2007) which are cytoplasmic damaged filaments, hyaline (glassy appearance) inclusions in hepatocytes of patients with alcoholic hepatitis, and were described over a century ago (Mallory 1911). The formation of these cytokeratin aggresomes can be prevented *in vitro* with an inhibitor against the kinase p38 which phosphorylates K8 at S74 (Nan et al. 2006). K8 S74 phosphorylation by p38 is the most prevalent PTM on keratins, and occurs in organs and cultured cells during cell stress, mitosis and apoptosis, and regulates keratin filament reorganization (Ku et al. 2002). Since keratins are highly abundant in epithelial tissue it allows a suitable role for keratins as targets of phosphorylation by stress-activated kinases (Omary et al. 2009). K8 S74A mutation predisposes to liver injury showing a molecular link between K8 phosphorylation and a disease-associated mutation (Ku and Omary 2006).

During particular cellular stress environments the expression of SEKs is enhanced up to 3-fold, which may partly explain the protective roles that K8 and K18 mediate in the liver (Omary et al. 2009). K8 potentially acts as “phosphate sponge” that absorbs p38 activity and therefore prevents apoptosis (Haines and Lane 2012). Keratin expression is stress-inducible even though keratins only account for 0.2-0.5% of all cellular proteins in hepatocytes and enterocytes (Omary 2017).

Keratins are also targets of hypersumoylation in an injury-dependent manner. K8/K18/K19 are sumoylated by poly-SUMO2/3 chains affecting filament organization and stimulus-induced keratin solubility (Snider et al. 2011). During basal conditions SUMO conjugation to keratins is of low level, but during oxidative stress, apoptosis, and during amount imbalance of type I and II keratins (occurs in certain liver injuries) sumoylation of keratins is considerably increased (Snider and Omary 2014). Hence, even the substitution of one particular amino acid on keratins can alter their PTMs and can lead to interruption of cellular function in different ways (Haines and Lane 2012). All in all, keratins as well as other intermediae filament proteins are considered in an increasing manner as stress proteins that protect cells and tissues from cell stress and injury (Toivola et al. 2010).

3.5 Keratins in disease

During alcohol withdrawal the amount of caspase-cleaved K18 is elevated, which can be utilized for prediction of liver-related deaths in humans with alcoholic liver disease (Mueller et al. 2017). K18 deficiency results in steatohepatitis-associated liver carcinogenesis (Bettermann et al. 2016) whereas K8 and K18 are upregulated as a consequence of injury in the pancreas (Omary et al. 2009). It has also been shown that K8 Y54H and G62C human mutations weaken keratin filament reorganization during heat stress, oxidative stress or exposure to okadaic acid (Ku et al. 2001). Mutations in keratins in the liver are silent (mutation not leading to amino acid change) in most humans, but carry diminished capabilities to resist liver injury mediated by cell stress in the form of toxins or viruses (Omary et al. 2009). When K8 and K18 are overexpressed in cancer the consequences are for example chemoresistance (Bauman et al. 1994; Anderson et al. 1996; Zhang et al. 2016). Repressed K8 levels also sensitize mice to streptozotocin-induced diabetes (Alam et al. 2018). For further details about the K8-null mouse phenotype caused outcomes in the intestine, please see section “3.3 Keratins in the colon”.

Keratin-encoding genes carrying aberrant sequences disrupt the structure, organization and/or regulation of keratin filaments and have been shown to cause for instance cell fragility disorders of the skin in the epidermis (Omary 2017) highlighting the importance of keratins in maintaining mechanical stability in tissues (Haines and Lane 2012). The importance of keratins for tissue resilience was first shown by identifying K5 and K14 mutations as causative pathogenic mutations in EBS (Bonifas et al. 1991).

Keratins carrying mutations may not behave improperly only in terms of cellular fragility or when facing cellular stress, but these mutations also pose a high risk for the buildup of chronic, acute and even rare medical conditions whose physiology show impaired cellular integrity and tissue homeostasis (Jacob et al. 2018). For instance, changes in normal encoding sequences of K8 and K18 genes increase the risk of acute and chronic liver diseases. Overexpression of SEKs as a consequence of injury has been detected for example in mouse liver, pancreatitis and cholelithiasis (gallstones), and human primary biliary cirrhosis which is nowadays known by the name primary biliary cholangitis (Omary et al. 2009; Floreani and Mangini 2018). Regarding colorectal cancer, repressed levels of K8 and K20 indicate the presence of an ongoing epithelial-mesenchymal transition (EMT) which is one of the most essential steps in the development of aggressive colorectal cancers

(Knösel et al. 2006). On top of this, K8 phosphorylation loss has been detected in the oral squamous cell carcinoma (OSCC) cell line AW13516 (Alam et al. 2011).

Already in the 1990's it was known that the gastrointestinal tract's simple/single-layered epithelium in which carcinomas often develop, consist mostly of intermediate filaments of specifically K8/K18. Carcinomas are defined as a category of cancer types that develop from epithelial cells (Oshima et al. 1996). The exact pattern of keratin expression which is maintained stable in neoplasms, is often used to reveal the origin of the primary tumor when studying samples of metastases for pathological diagnosis. For example, hepatocellular carcinomas normally express K8 and K18 whereas colorectal carcinomas normally express K20, but not K7. On top of this, the keratin expression patterns can be used as prognosis when predicting the outcome of breast cancer where K8 and K18 expression predicts improved survival, or of colorectal carcinomas where low K20 expression correlates with favorable survival rate (Omary et al. 2009; Floreani and Mangini 2018).

AIMS OF THE THESIS

Notch is a critical regulator of stem cell differentiation with dose- and context-dependent outcomes. In order to gain more knowledge on how Notch activity is fine-tuned in different cellular and developmental contexts, we are in need of identifying Notch regulatory proteins and mechanisms.

The specific aims of this thesis were:

1) To elucidate mechanisms and consequences of Notch sumoylation during cell stress.

2) To elucidate the interaction of Notch with the cytoskeletal protein keratin and the consequences of this interaction for stem cell differentiation and tissue homestosasis.

EXPERIMENTAL PROCEDURES

The experimental procedures used in this thesis are listed in the following tables. More detailed information about specific methods are described in the original publications (I-II).

Cell lines

Name	Type	Original work
HeLa	Human cervical carcinoma	I.
COS-7	African green monkey kidney	I.
HEK 293 FLN	Human embryonic kidney cells overexpressing FLN	I-II.
Mef Vim$^{-/-}$	Mouse embryonic fibroblast from vimentin knock-out mice	II.
Caco-2	Human colorectal adenocarcinoma	II.
Caco-2	Human Caco-2 CRISPR/Cas9 K8$^{+/+}$, K8$^{+/-}$, K8$^{-/-}$	II.
MCF7	Human breast cancer	II.

Mice

Name	(Background)	Original work
K8$^{+/+}$	(FVB/n)	II.
K8$^{+/-}$	(FVB/n)	II.
K8$^{-/-}$	(FVB/n)	II.

Plasmids (human)

Name	Original Work
ΔEN	I-II.
GFP-Flag-NICD	I-II.
GFP-Flag-NICD K1774/1780/1781/1782R	I.
His-SUMO1	I.
His-SUMO2	I.
His-SUMO3	I.
SENP1	I.
SENP2	I.
Flag-CSL	I.
Flag-HDAC4	I.
Myc-NICD	I.
FLN	II.
K8	II.
KS74A	II.
K18	II.
K19	II.
PCMVKM	I.
PCDNA3.1	I-II.
12xCSL luciferase reporter	I.
β-galactosidase	I.

Methods

Name	Original work
Alcian blue staining	II.
Antibiotic treatment	II.
Cell culture	I-II.

CRISPR/Cas9 deleted K8 gene	II.
Chromatin immunoprecipitation ChIP, modified	I.
Cycloheximide treatment	I-II.
Extraction of nuclear and cytoplasmic cell fractions	I.
High-salt extraction	II.
Image analysis	I-II.
Immunofluorescence (IF)	I-II.
Immunohistochemistry (IHC)	I-II.
Immunoprecipitation (IP)	I-II.
In ovo electroporation	I.
Luciferase assay	I.
MG 132 treatment	II.
PCR for mouse genotyping	II.
Periodic acid-schiff (PAS) staining	II.
Proximity ligation assay (PLA)	II.
RNA extraction and quantitative RT-PCR	I-II.
RNA interference	I.
SDS-PAGE and immunoblotting	I-II.
Site-directed mutagenesis	I.
Statistical analysis	I-II.
Structural modeling	I.
Transient transfections	I-II.

Antibodies

Name	**Company**	**Application**	**Original work**
Alexa Fluor 488	Invitrogen	IF	I-II
Alexa Fluor 546	Invitrogen	IF	II.
β-actin	Cell Signaling	WB	I-II.
Cleaved Notch Val-1744	Abcam	WB, IHC	I-II.
Cox1	Santa Cruz	PLA	II.
Flag	Sigma-Aldrich	WB, IP	I.
GFP	Invitrogen	WB	I.
HDAC4	Abcam	WB, IP	I.
Hey1	Santa Cruz	IF	II.
Histone H3	Abcam	WB	II.
Hsc70	Stressgen Bioreagents	WB	I-II.
K8 (273)	gift of J. Eriksson lab	WB, IP, IF	II.
K8	Progene	PLA	II.
K8, Troma I	Dev.Hyb.Bank, Uni.Iowa	WB, IF	II.
K18 (275)	gift of J. Eriksson lab	WB, IP, IF	II.
K18 (L2A1)	gift of B. Omary lab	IP	II.
K19	Sigma-Aldrich	WB	II.
Notch C-20 FLN	Santa Cruz	WB, IF, IP, PLA	I-II.
Notch1	Thermo Scientific	IF	II.
Phosphohistone H3	Cell Signaling	WB, IF	II.
SUMO1	Zymed Laboratories	WB, IP	I.
SUMO2/3	Zymed Laboratories	WB, IP	I.
Synaptophysin	Abcam	WB, IF	II.
Villin	Abcam	WB	II.

RESULTS AND DISCUSSION

1. Sumoylation of Notch1 regulates its target gene expression

The data discussed in chapter 1 of the results and discussion section is part of aim 1 and presented in the publication Antila C et al., Sumoylation of Notch1 represses its target gene expression during cell stress. Cell Death Differ 25:600-615, 2018.

This project provided new insights into Notch signaling, and how transcriptional activity of Notch is controlled as a consequence of sumoylation during cellular stress. Notch is a signaling mechanism critically important for most cell fate choices, tissue patterning and differentiation programs. Despite the molecular simplicity in design, Notch signaling is pleiotropic and affects cellular function in a context-dependent manner (Aster et al. 2017). Notch target genes are expressed in a cyclic manner and the phenotypic outcome of Notch signaling is critically dependent on signaling dose. Hence, the Notch pathway must be able to elicit appropriate responses in distinct cellular contexts, and fine-tune Notch transcriptional activity during different physiological situations, but how this is achieved has not been well understood (Imayoshi et al. 2013). Complex layers of PTMs have been suggested to fine-tune Notch signaling. The transcriptional activity and turnover of Notch are heavily impacted by PTMs of Notch (Lee et al. 2015). The identification of site-specific modifications and a deeper understanding of how Notch is regulated at the level of PTMs is a major challenge of current research.

Project (I) characterized, at many levels of the Notch signaling cascade, a PTM of NICD, which influenced Notch target gene expression by the NICD-CSL-MAML transcriptional complex. Specifically, project (I) described that site-specific sumoylation of NICD seemed to facilitate recruitment of HDAC4 to the NICD-CSL-MAML complex on chromatin to repress target gene activation without permanently ending the Notch signal by NICD degradation (Fig. 7A-J). The experimental evidence was further supported by molecular modeling of the sumoylation event in the transcriptional complex (Fig. 4, SI Fig. 4). We provided substantial evidence verifying that Notch is indeed sumoylated on the identified site (Fig. 5A-C, G-I, K, Fig.7E-J) and demonstrated the significance of Notch sumoylation on Notch target gene expression in cells (Fig. 5D-K, Fig. 7H-J) and in the developing central nervous system (CNS) of chick embryos subjected to cellular stress (Fig. 6, SI Fig. 5-6). Importantly, we showed that sumoylation was context-dependent and enhanced during cell stress (Fig. 2C-D, SI Fig. 1A, SI Fig. 2C, SI Fig. 3C) which shed light on how canonical Notch signaling is dynamically modified during physiological stress conditions.

Recent data highlights sumoylation as a key mechanism for PTMs in cancer progression, cell proliferation, metabolism, inflammatory signaling and development (Ding et al. 2016; Du et al. 2016; Hendriks and Vertegaal 2016; Lee et al. 2016; Streich and Lima 2016). The identification of Notch, a key regulator of tissue development and homeostasis, as a new target for sumoylation affecting several aspects of the Notch signaling pathway, was an important contribution to the field. Notch is an addition to the growing body of SUMO substrates which are involved in cell fate specification during development.

1.1 The intracellular domain of Notch is sumoylated

Developing new tools for the identification of yet undiscovered SUMO substrates has led to an increase not only in the number of sumoylation target proteins, but also in the knowledge of how SUMO proteins bind to their specific substrates. Before starting project (I), Notch had been identified as a putative substrate for SUMO2 under conditions of cellular stress in two separate screens. The first screen was based on purification and identification of novel SUMO2 substrates in heat shocked cells. More specifically, the screening was based on the desumoylation of substrates prior to SDS-PAGE using a recombinant SUMO protease to reduce the complexity of the sample for identification in LC-ESI-MS/MS. Using this approach Notch1 was identified as a putative SUMO substrate (Blomster et al. 2009).

Prior to initiating project (I), another screening approach was subsequently developed, based on identifying sumoylation sites by a signature tag and cysteine-targeted affinity purification which indicated that Notch undergoes conjugation by SUMO in the RAM domain within a sequence that contains the four amino acids K1774, K1780, K1781 and K1782 (Blomster et al. 2010). In this thesis we focus on the verification of this sumoylation site and its impact on various outcomes of Notch signaling. If not stated otherwise, all the Notch constructs mentioned in discussion of project (I) results refer to Notch1 specifically, and all SUMO constructs if not stated otherwise refer to SUMO1 specifically.

The non-consensus lysine residues located at positions 1774, 1780, 1781 and 1782 on Notch1 are evolutionarily conserved in several model species (Fig. 1), which suggests an important role for this sequence. Based on the mentioned screens (Blomster et al. 2009, 2010) we assessed if Notch1 is subject to SUMO binding. Overexpression of GFP-Flag-NICD and His-SUMO1 in HeLa cells revealed monosumoylation of NICD in the immunoprecipitated (IP) sample at a molecular weight between 150 and 250 kDa. The lysate did not show sumoylated NICD, only unsumoylated NICD just below the 150 kDa marker. The reason that NICD sumoylation was detected only in the immunoprecipitate (IP sample) and not lysates might be the presence of isopeptidases desumoylating Notch in the lysates, whereas in the IP sample the deconjugating proteins were washed away. Another contributing factor, considering that normally only a small fraction of any given protein is subject to sumoylation, may also be that in the IP sample, NICD was concentrated and the entire IP sample was analyzed for SDS-PAGE, whereas the lysate was diluted in 3x Laemmli lysis buffer and consequently less NICD was analyzed on SDS-PAGE. One reason for lysing lysate samples directly in Laemmli lysis buffer followed by boiling of samples is that the lysis is supposed to happen so fast that the SUMO-deconjugated enzymes will not have time to function, but there was still a difference of observable NICD sumoylation between the lysate and IP sample (Fig. 2A). Sumoylation of Notch was, although very weakly, detected also when the membrane-tethered form of Notch ΔEN, instead of the NICD, was overexpressed (Fig. 2B).

1.2 Cellular stress enhances Notch sumoylation

Cellular stress has been reported to be able to enhance sumoylation, a phenomenom known as the SUMO stress response (Enserink 2017). How enhanced sumoylation followed by cell stress occurs mechanistically is not known, but an emerging view recognizes cell stress modifying desumoylating enzymes (Guo and Henley 2014). It has been suggested that all SENPs except SENP6 are sensitive to **heat stress** which is a form of proteotoxic stress which in turn refers to any impairment of cell function caused by misfolding of a protein. Heat shock also decreases the ratio of sumoylated to unsumoylated E1 SAE2. The unsumoylated SAE2 efficiently transfers SUMO to Ubc9 and enhances final substrate sumoylation (Guo and Henley 2014). The degradation of SENPs during cellular stress does not apply only to heat shock. It has been shown that during brain ischemia, SENP3 undergoes degradation as a consequence of **oxygen/glucose deprivation** leading to global increase of SUMO2/3-conjugation (Guo et al. 2013). In addition, in the plant *Arabidopsis Thaliana*, the degradation of the SUMO-deconjugating proteins Overly tolerant to salt1 and -2 (OTS1 and OTS2) increases SUMO1/2-conjugated proteins in response to **osmotic stress** (NaCl) (Conti et al. 2008). A more recent study provided emerging evidence for **hypoxic** inactivation of SUMO-specific isopeptidases (Kunz et al. 2016). It has also been hypothesized that chaperones sequester unconjugated SUMO during normal conditions in the cytoplasm, and that these SUMO isoforms are released and mobilized during a particular cellular stress condition (Enserink 2017). Other cell stresses known to enhance sumoylation include for instance **nutrient starvation**, endoplasmic reticulum (**ER) stress**, **oxidative stress** (H_2O_2, NO) and **genotoxic stress** (including ionizing radiation, UV exposure, and chemotherapeutic reagents such as etoposide and doxorubicin) that generates DNA double strand break (Peek et al. 2018).

When HeLa cells were heat shocked, Notch sumoylation by both SUMO1 and SUMO3 increased drastically. Several proteins were detected between 150 and 250 kDa (Fig. 2C). The most efficient sumoylation of Notch was detected in cells exposed to a 1-hour heat shock at 42°C followed by a 1-hour recovery at 37°C. Sumoylation was transient and was no longer detected after 3 hours of recovery from heat stress (SI Fig. 2C). In Fig. 2B the signal for sumoylation was weak, yet detectable. In this experiment no cellular stress was used once again emphasizing the essential role of stress in Notch sumoylation. Sumoylation was challenging to detect in the absence of cellular stress also when NICD was overexpressed (Fig. 2A).

As cellular stress in the form of heat shock seemed to enhance NICD sumoylation, we studied the impact of other forms of cellular stresses intrinsic to the cellular environment. The synthesis of proteins is sensitive to mistakes. It has been estimated that as many as 30% of proteins are defectively folded and thereby degraded by the proteasome within minutes of their synthesis. If misfolded proteins aggregate and accumulate in the lumen of the ER it will cause increased basal proteasome load (ER stress) that will, if not counteracted, lead to global malfunction of cellular function and ultimately to cell death (Guang et al. 2019). **Cell stress in the form accumulation and aggregation of misfolded proteins (ER stress) can be enhanced by inhibiting the proteasome** (Guang et al. 2019). We broadened the concept of cellular stress in Notch sumoylation by concentrating on how cell stress induced by the proteasome inhibitor bortezomib affects SUMO conjugation to Notch, and observed a remarkably enhanced sumoylation of Notch (Fig. 2D). Bortezomib has been approved by

the U.S. Food and Drug Administration (FDA) for the treatment of many different cancers (Sterz et al. 2008). Other FDA approved proteasome inhibitors include carfilzomib and ixazomib (Guang et al. 2019). Recently almost 15,000 endogenous SUMO2/3 sites were identified in human cells during heat stress and proteasomal inhibition using a peptide-level immunoprecipitation enrichment strategy (Hendriks et al. 2018) highlighting the effect of these two cellular stress conditions on sumoylation.

We did not utilize SUMO4, but when human embryonic kidney 293 (HEK293) cells are subjected to stress in the form of serum starvation, the cells mediate SUMO4 maturation, and SUMO4 is able to conjugate to its substrates (Wei et al. 2008). Notch contributes to the development of CNS-related diseases (literature section 1.6.1 "Notch in disease in the CNS"). Sumoylation in turn acts as a neuroprotective response mechanism against drastic stress. It is known that global levels of sumoylation are heavily increased in the hibernating ground squirrel brains suffering from hypoxia/glucose deprivation, showing that sumoylation has a cytoprotective role against these two stresses (Lee et al. 2007). Additionally, it has been shown that loss of SUMO2/3 decreases survival of osteosarcoma U2OS cells after heat shock, and survival of primary cortical neurons after oxygen/glucose deprivation (Guo and Henley 2014). In accordance, increased global sumoylation in Ubc9 transgenic mice confers resistance to brain ischemia (Lee et al. 2011).

What directs the sumoylation stress response is not known. DNA repair proteins become sumoylated as a consequence of DNA damage, but the same proteins do not undergo sumoylation by other stresses such as nutrient stress (Enserink 2017). In this thesis we raised the question whether Notch activity is influenced by stress to the cells. Our approach was to investigate whether Notch is regulated by sumoylation and whether this in turn is altered when cells are subjected to heat shock or proteasome inhibition stress. Our results showed that NICD sumoylation was enhanced when cells were subjected to either one of the mentioned cellular stress forms (Fig. 2C-D, SI Fig. 1A, SI Fig. 2C, SI Fig. 3C) and that these modifications impacted on Notch function in different ways. The role that cellular stress mediates on Notch signaling via sumoylation may have long lasting physiological effects. Because Notch has such a central role in regulating cell fate choices during development, an effective and accurate modification of Notch signaling may have potential significance to direct stem cell differentiation. Understanding how Notch is regulated by environmental stimulus is also of uttermost importance for cancer research as Notch activity regulation by cellular stress intrinsic to the tumor environment or occurring as a consequence of therapy might provide the cells with survival mechanisms that drive further tumor progression, therapy resistance and recurrence. Such knowledge will help to rationally drug the disease.

1.3 Interaction between Notch and endogenous SUMO

In order to determine which SUMO isoforms of endogenous origin have the capacity to bind to Notch, HeLa cells with overexpressed Notch were immunoprecipitated against Notch and immunoblotted either against endogenous SUMO2/3 for which a signal was detected (Fig. 2E), or against endogenous SUMO1 for which a signal was not detected (Fig. 2F, SI Fig. 2D).

A noticeable feature in the nature of experiments in project (I) is that in many experiments overexpressed SUMO1 was used instead of overexpressed SUMO2/3 despite that we were not able to detect endogenous SUMO1 binding to Notch (Fig. 2F). However, we could not rule out that for SUMO1 it was simply a question of challenging detectability. It has been shown that the amount of both total and free SUMO2/3 is larger than that of SUMO1 (Saitoh and Hinchey 2000), which simply might make it easier to detect endogenous SUMO2/3 versus SUMO1. Overall, SUMO1 seems to be involved mostly in regulating cell physiology and maintenance whereas SUMO2/3 mostly have roles in cell stress responses (Guo and Henley 2014). However, SUMO1 has been a model SUMO-isoform to be widely used within the SUMO society.

We demonstrated that overexpressed SUMO1 and SUMO2 as a cause of cellular stress bound to Notch (Fig.2G) and that this interaction can be abrogated with the overexpression of the de-sumoylating proteins SENP1 and SENP2 (Fig. 2G). SENP1 and SENP2 have the capability of desumoylating the substrates of SUMO1 and SUMO2/3 whereas the other SENP-isoforms seem to be more selective for SUMO2/3 (Sarge and Park-Sarge 2009).

Without overexpressed SUMO, NICD sumoylation is challenging to detect as can be seen in Fig. 2E-F and SI Fig. 2A. Adding to the challenge of SUMO detection is most likely the presence of isopeptidases in the cell lysates/whole cell extracts deconjugating SUMO from its target proteins. The actions of SUMO-deconjugating proteins can be fought for instance with N-ethylmaleimide (NEM) that inhibits desumoylation. In addition to sufficient SUMO overexpression, NEM, cellular stress, more advanced SUMO antibodies, and also more sensitive and specific Notch antibodies are needed in optimizing Notch sumoylation detection due to the short-lived nature and low abundance of Notch.

The IP experiments in Fig. 2 are gaining significance from SI Fig. 1B which shows SUMO conjugation to endogenous Notch. In SI Fig. 1B we immunoprecipitated Notch and immunoblotted first against SUMO and detected the modified form of endogenous Notch. Reblotting against Notch identified the modified Notch at around 150 kDa as well as the unmodified form of Notch closer to 100 kDa. In SI Fig. 1A we used human embryonic kidney full length Notch cells (HEK 293 FLN cells), a cell line that overexpresses full length Notch. Notch was activated with Notch-specific recombinant ligands immobilized on the cell culture substrate. The results showed that Notch activated with Notch ligands can be sumoylated as NICD, and more efficiently in the presence of heat shock. This system was quite robust and was employed to further confirm the biological significance of SUMO conjugation to Notch (SI Fig. 1A).

Sumoylation is a rapid, dynamic, reversible process in which many rounds of sumoylation are often needed to elicit a detectable biological response (Gill 2004), and a process in which most SUMO target proteins are not sumoylated at a random time point (Hwang and Kalejta 2011). In order to analyse the effects of Notch sumoylation, a substantial amount of the experiments was performed with overexpressed proteins, since detection of endogenous SUMO binding to Notch was generally challenging and allowed us to use overexpression of sumoylation deficient variants of Notch.

1.4 Stress-inducible sumoylation of Notch occurs in the nucleus

SUMO is primarily located in the nucleus (Takahashi et al. 2008) and consequently sumoylation has been described mostly for proteins residing in the nucleus regulating especially transcription, genome integrity and nuclear structure (Guo and Henley 2014). We next evaluated the cellular localization where Notch undergoes sumoylation, and whether sumoylation regulates the nuclear enrichment of Notch. Therefore, we assessed, by immunofluorescence confocal microscopy, whether stress-induced sumoylation of Notch alters the nuclear localization of NICD (Fig 3A-B, SI Fig. 3A-B). Cells were analyzed based on if Notch localization was strongly nuclear, equally nuclear/cytoplasmic or mostly cytoplasmic. Stress-induced sumoylation of NICD seemed to increase the nuclear localization of NICD. The strongest nuclear localization of NICD was observed in heat shocked cells that were overexpressed with SUMO1 and ΔEN1. 58% of these cells displayed strong nuclear staining whereas only 41% of the heat shocked cells that overexpressed ΔEN1, but not SUMO1, showed strong nuclear staining. Approximately 37% of the cells that were not heat shocked, but overexpressed Notch and SUMO, showed strong nuclear staining. This indicates that both SUMO and cellular stress in the form heat shock enhance Notch nuclear localization mostly when both of these factors are combined.

We decided to not use an overexpressed NICD construct in this experiment (Fig 3A-B, SI Fig. 3A-B), but ΔEN instead. By using the membrane tethered ΔEN which after intramembrane cleavage generated NICD that migrated to the nucleus, we received information about how Notch sumoylation affects different steps in the Notch signaling pathway and further verified the results obtained with experiments performed with overexpressed NICD. As ΔEN is activated by proteolysis it comprises also the proteolytic activation step in the Notch signaling cascade and is subject to more levels of regulation than only the activated form NICD.

This analysis (Fig 3A-B, SI Fig. 3A-B) might be confounded by the presence of endogenous Notch1, but the negative control with overexpressed vector pcDNA3.1 showed negligible expression of endogenous active Notch. Therefore, the experiment (Fig. 3A-B) did not give insights about the role of SUMO conjugation on endogenous Notch. Only a small amount of NICD was detected in the nucleus in the control samples since sparse non-confluent cell culture showed only little endogenous activation of Notch as activation requires cell-cell contact, and the turnover of NICD was rapid due to dose-dependence of Notch. NICD levels are tightly regulated, occur in minute amounts while high amounts suppress cell function (Mazzone et al. 2010) and it is difficult to study PTMs with endogenous proteins. CRISPR/Cas9 and endogenous site-specific modifications of specific PTM sites can give insight into the functional consequences although biochemistry is still difficult due to lack of proper tools (poor antibodies), a recognized problem in the field.

The purpose of stress-induced sumoylation of NICD to increase nuclear localization of NICD can be speculated. Heat shocked cells with overexpressed NICD and SUMO1 showed more nuclear localization of Notch than when either SUMO1 overexpression or heat shock alone (Fig 3A-B, SI Fig. 3A-B). Sumoylation of NICD in the nucleus (Fig 3C-D) as a consequence of cellular stress might retain Notch viable in the nucleus and thus protect it from being degraded. As we showed that stress-induced sumoylation enhances NICD levels (Fig. 3E-F, SI Fig. 3C-E), specifically enhanced nuclear NICD levels by

stress-induced sumoylation could be a potential mechanism to protect Notch from degradation and thus maintain Notch stability during stress situations so that the Notch signal does not have to be activated with new ligands when the acute stress situation is over. That Notch was sumoylated only in the nucleus (Fig. 3C-D) and that SENP1 and SENP2 deconjugated Notch (Fig. 2G) are also in line with SENP1 and SENP2 showing distributions at subnuclear compartments (Chow et al. 2014). The following section focuses more prevalently on the effect of sumoylation on Notch levels.

1.5 NICD levels are increased by SUMO

Changes in protein levels have been reported for a variety of SUMO substrates (Geiss-Friedlander and Melchior 2007). Therefore, we addressed the effect of sumoylation on levels and degradation of Notch. We showed that heat stress-induced sumoylation considerably elevates Notch levels. This was demonstrated in several settings with and without the presence of heat shock and SUMO, by inhibiting protein synthesis for different time periods with the protein translation inhibitor cycloheximide (CHX), followed by consequent observation of Notch levels (Fig. 3E, SI Fig. 3C and D). In addition, we demonstrated that Notch levels increased upon sumoylation in a dose response experiment where Notch levels correlated positively with the amount of overexpressed SUMO (Fig. 3F, SI Fig. 3E). Heat shock also resulted in higher Notch sumoylation and higher levels of the unmodified form of Notch (SI Fig. 3C). The half-life of NICD is 180 minutes (Fryer et al. 2004), which allows for its concentration to respond fast to alteration in activation of Notch (Nandagopal et al. 2018).

Of note, unmodified NICD levels increased when SUMO was overexpressed (Fig. 3E-F, SI Fig. 3C-E), indicating that overexpression of SUMO might result in increased NICD abundance through a mechanism that does not involve sumoylation of NICD itself (i.e. not direct). Further, Fig. 3C shows that overexpressed SUMO leads to stabilization of NICD in the cytoplasm which is devoid of sumoylated NICD, and therefore overexpression of SUMO perhaps has consequences, that are not related to sumoylation of NICD itself. However, since Notch was sumoylated in the nucleus (Fig. 3C-D) and since a very dominant portion of detected unmodified form of Notch in Fig. 3C-D was localized in the nucleus, it would indicate that SUMO overexpression affects mostly nuclear Notch and not cytoplasmic Notch. Also, SUMO1, SUMO2/3, Ubc9 and E1 SAE1/SAE2 are mainly nuclear (Jürgen Dohmen 2004). Therefore, enhanced nuclear levels of Notch cannot most likely be explained by a single hypothetical cytoplasmic event. Additionally, cytoplasmic fractions in Fig. 3D, regardless of presence or absence of overexpressed SUMO, showed the same degree of NICD levels indicating that SUMO overexpression did not affect cytoplasmic NICD levels in any way. Overexpression of SUMO also resulted in enhanced nuclear localization of Notch, and enhanced levels of NICD (Fig. 3E-F, SI Fig. 3C-E) when Notch was overexpressed in the form of the membrane tethered ΔEN (Fig. 3A-B). Further, the levels and the nuclear localization of a sumoylation-deficient TSM (tetra SUMO mutant) were also lower than those of WT NICD when SUMO was overexpressed (Fig. 5A). Stress-induced sumoylation increased the nuclear localization of Notch (Fig. 3A-B, SI Fig. 3A-B). In the presence of SUMO and heat stress, WT Notch showed stronger nuclear accumulation compared to the sumoylation deficient TSM (Fig. 5B), which is also in line with the notion that stress-induced sumoylation enhances nuclear NICD levels. The results

suggest enhanced levels of Notch as a consequence of stress-induced sumoylation. However, the experiments do not rule out indirect effects of SUMO on Notch levels, due to for instance SUMO-mediated regulation of the activity of Notch ubiquitin ligases or kinases that mark Notch for ubiquitin-mediated degradation.

Others (Vennemann and Hofmann 2013) have shown that overexpression of SUMO may lead to overall increased sumoylation, which may have many non-specific effects. Such non-specific effects could potentially be for instance elevated SUMO interfering with the proteasome leading to general stabilization of proteins. We wanted to rule out non-specific effects from sumoylation and therefore we included additional controls. To determine whether the increased NICD levels were specific to stress-induced sumoylation of Notch and not due to general stabilization of proteins, another labile protein HIF-1α which shows extensive cross-talk with Notch (Gustafsson et al. 2005; Sahlgren et al. 2008; Main et al. 2010) was analyzed. The levels of HIF-1α were not increased by heat shock and overexpression of SUMO by immunoblotting whereas the levels of Notch were (Figure 28, not included in published article of project (I)). All in all, many pieces of data suggest that enhanced Notch levels detected in Fig. 2D, 3E-F, SI Fig. 3C-E, were a consequence of SUMO on Notch itself rather than SUMO overexpression mediating enhanced Notch levels via other mechanisms.

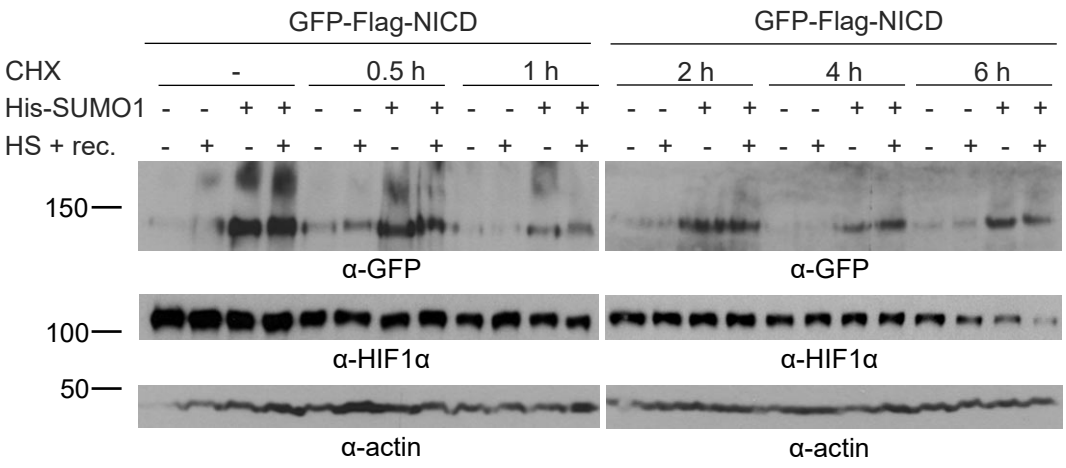

Figure 28. In contrast to Notch levels, HIF1α levels are not increased as a consequence of heat shock treatment and SUMO overexpression.

1.6 SUMO binds to NICD in the RAM domain

Screening experiments (Blomster et al. 2009, 2010) performed prior to initiation of project (I) had suggested that Notch1 is not only a potential target of undergoing conjugation by SUMO, but also that the site where this specific binding occurs, is located within a sequence of the RAM domain consisting of four lysine residues K1774, K1780, K1781 and K1782. This part of the RAM domain binds to the transcription factor CSL during canonical Notch

signalig (Kopan and Ilagan 2009; Wang et al. 2015). Since sumoylation of NICD was shown to take place in the nucleus (Fig 3C-D) it was of interest to determine if the conjugation of SUMO to NICD was possible to occur in the transcriptional activation complex NICD-CSL-MAML. The structure of the Notch transcriptional complex has been solved earlier (Nam et al. 2006; Wilson and Kovall 2006).

A three-dimensional model showed that the lysines K1780, K1781 and K1782 within the RAM domain reside on the surface of the NICD-CSL-MAML transcriptional complex. The model further elucidated that the lysines are easily accessible to sumoylation without affecting the RAM domain-CSL interaction or any other component within the NICD-CSL-MAML transcriptional complex. Additionally, neither SUMO or the SUMO-conjugating protein Ubc9, interfere with the binding of NICD to CSL (Fig. 4, SI Fig. 4). These findings were essential in gaining a broader understanding of the concept of how Notch functions at the level of the chromatin. These discoveries also provided important insights considering ways to interfere with potential hyperactivity of the pathway in case of disease. Therefore, we mutated the four lysines and created a K1774/K1780/K1781/K1782R GFP-Flag-NICD which we called TSM.

As further demonstrated and discussed in section "1.9 Sumoylation on the RAM domain does not block RAM-CSL interaction" sumoylation does not affect the interaction between NICD and CSL (Fig. 7).

Since the three-dimensional model (Fig. 4, SI Fig. 4) supported the results of the screening experiments of Notch being a sumoylaton target protein and the site being inside a short specified sequence inside the RAM domain (Blomster et al. 2009, 2010) we tested if the TSM is able to undergo sumoylation. It turned out that in heat stress conditions, only WT Notch was conjugated to SUMO whereas the TSM was not, both in lysates and co-immunoprecipitates, supporting the notion that the mentioned short sequence within the RAM domain is a sumoylation site in Notch (Fig. 5A).

1.7 Sumoylation of Notch represses its target gene expression

Although studies done with mammalian cells and budding yeast have provided evidence for sumoylation being capable of increasing transcription activity in some cases, SUMO conjugation to transcription factors as a result of cell stress most often results in a repressive effect in terms of transcriptional activity (Enserink 2017). For instance, sumoylation of Forkhead Box P1 (FOXP1) promotes transcriptional repression by enhancing binding of FOXP1 to the co-repressor Ctbp1 driving dendritic morphogenesis (Rocca et al. 2017).

Activation of Notch leads to the nuclear translocation of the activated receptor and activation of the Notch transcriptional complex. Hence, transcriptional activation and expression of Notch target genes as measured by reporter assays or RT-PCR are widely used as measures of Notch activity (Enserink 2017). To further understand the role of sumoylation and the TSM in the Notch pathway, we used a luciferase reporter gene reflecting Notch activity and detected increased Notch activity with the TSM compared when using WT Notch. The difference in detected Notch activity between WT Notch and the TSM was greater when the cells were subjected to heat stress (Fig. 5C) indicating that

the difference in Notch activity, between WT and the TSM occurred via sumoylation, and that sumoylation decreased Notch transcriptional activity.

That sumoylation seemed to decrease Notch transcriptional activity (Fig. 5C) led us to study the mRNA levels of classical Notch target genes Hes1, Hey1 and Hey2, by RT-PCR. We chose to focus on the members of the Hes and Hey gene family. Especially in mammalians the most studied Notch target genes are the target gene members Hes1, Hes5 and Hey1 all of which have, when being dysregulated, been shown to clearly be drivers of tumorigenesis and further evolvement of tumors (Lee et al. 2015). These genes are essential regulators of development in many organs where they expand progenitor cell pools and steer decisions regarding cell fate (Kobayashi and Kageyama 2014).

1.7.1 Notch sumoylation decreases expression of Notch target genes during cell stress

Cellular processes during development require adaptable responses at the level of gene expression (Bigas and Espinosa 2018). Since Notch sumoylation seemed to decrease Notch transcriptional activity (Fig. 5C) the potential repressive effect of sumoylation on the expression of Notch target genes Hes1, Hey1 and Hey2 was examined by studying the effect of SUMO on mRNA levels of the mentioned Notch target genes. Of note, experiments with genetically modified mice have shown that different Notch isoforms partially have different target genes (Chari and Winandy 2008). We have in this project studied only the Notch1 isoform. During heat stress, SUMO overexpression repressed the expression of the Notch target genes Hes1, Hey1 and Hey2. Addition of SUMO decreased the expression of each studied target gene regardless of the recovery time after heat shock (Fig. 5D-F). One of the main aims of this project was to conclude how NICD sumoylation alters the Notch signaling pathway and what the most prominent and most important biological changes are that the pathway experiences as an outcome of NICD sumoylation. One of these features, perhaps the most prominent one, appears to be the repression of Notch1 target gene expression during cell stress.

1.7.2 Heat shock itself regulates Notch target gene expression

A noticeable observation was that heat shocked cells (1 hour heat shock, no recovery time) without the overexpression of SUMO increased expression activity of Notch target genes Hes1, Hey1 and Hey2 (SI Fig. 2B). This is consistent with for instance the study observing that nutrient deprivation of ER stress induces TRB3/USP9-dependent Notch activation in cancer (Izrailit et al. 2017). Heat shock followed by recovery time increased sumoylation (Fig. 2C, SI Fig. 1A, SI Fig. 2C, SI Fig. 3C) and sumoylation in heat shocked cells followed by recovery time in turn decreased Notch target gene expression (Fig. 5D-F). This is possibly a question of the ratio of sumoylated Notch versus unsumoylated Notch. It may be that acute stress itself without recovery time enhances Notch target gene expression (SI Fig. 2B) through a mechanism that does not involve sumoylation. But when the stress condition is followed by recovery time, SUMO, especially as overexpressed, is potentially more capable of conjugating more efficiently to Notch.

The relation between Notch and cellular stress is complex. Notch is the fundamental oncogene in T-ALL which is characterized by too many lymphoblasts (immature white

blood cells) in the blood, bone marrow and tissues such as mediastinal lymph nodes. Recently it became evident that Notch hijacks the cellular stress response machinery in T-ALL. This occurs by Notch binding to the heat shock factor 1 (HSF1) promoter and activating expression of HSF1 which governs the non-oncogenic cellular stress response. HSF1 activity in turn is important for T-ALL evolvement in animal models. In addition, Notch also binds to the promoters of most HSF1 targets giving Notch a broader control over the cellular stress response machinery. The hijacking seems to happen only in oncogenic conditions since Notch does not bind to the HSF1 promoter for instance in hematopoietic stem and progenitor cells (HSPC) even during heat shock (Kourtis et al. 2018). This eludes questions whether stress-induced modifications of Notch in T-ALL oncogenic conditions could be, at least partly, responsible for diverging Notch to promoters to which Notch in normal non-oncogenic conditions does not bind to.

1.7.3 Notch target genes are differentially regulated by wildtype Notch and the sumoylation deficient form of Notch

In heat shocked cells in the presence of overexpressed SUMO, the TSM displayed higher expression activity of the genes Hey1 and Hey2, compared to WT Notch (Fig. 5G-I) providing more support to the RAM domain being an essential sumoylation site. The effect of the TSM in enhancing Hes1 gene expression was weaker than for Hey1 and Hey2. To broaden the understanding of cell stress in regulating Notch target gene expression via Notch sumoylation, we continued by elucidating the role of cell stress-induced sumoylation in regulating the activity of classical Notch target genes (Fig 5J-5K). Bortezomib-induced sumoylation repressed the expression of the Notch target genes Hes1, Hey1 and Hey2 in HEK 293 FLN cells in which Notch was not overexpressed by transfection, but only activated with immobilized Notch-specific ligands (Fig. 5J). We utilized this method that confirmed that Notch can be sumoylated in a more natural setting (Fig. 5J) confirming the biological significance in Fig. 5D-I.

The role of SUMO during cellular stress in the repression of Notch target genes was in addition to Fig. 5D-J, also shown in another experiment. Overexpression of SUMO in HeLa cells repressed expression of Notch target genes Hes1, Hey1, and Hey2 during cell stress in the form of bortezomib (Fig. 5K). In Hela cells we compared the effect of bortezomib-induced sumoylation of WT Notch and the TSM on the expression of the three mentioned Notch target genes. We saw that in the presence of bortezomib and SUMO, target gene expression was less repressed with the TSM than with WT Notch (Fig. 5K), indicating the same effects that were already observed in Fig. 5C. As a future experiment, it could be beneficial to detect repressed levels of Hes and Hey also at protein level, not only as mRNA levels (Fig. 5D-K) although lack of sensitive antibodies creates challenges in the detection of Hes and Hey proteins.

There might be specific genes that are Notch target genes only as a consequence of cell stress-induced Notch sumoylation. However, recent RNA-sequencing in which WT Notch versus the TSM in the presence and absence of heat shock, did not reveal such gene specificity (Sahlgren lab, unpublished results) at first glance. However deeper analyses of the results combined with analyses of promoter/enhancer occupancy of NICD is needed to

answer this question. The lack of major global effects on gene expression pinpoints a fine-tuning role for Notch sumoylation.

It was certainly an interesting finding that there was more nuclear NICD in the conditions of heat shocked cells being overexpressed with SUMO1 (Fig. 3A-B, SI Fig. 3A-B) when other findings revealed that the expression of all studied Notch target genes Hes1, Hey1 and Hey2 was dampened as an outcome of stress-induced sumoylation of Notch (Fig. 5D-K). The finding of highly stable NICD and repressed expression of target genes may be related to "transcriptional switching" e.g the possibility to switch Notch transcriptional activity on and off without relying on ligand mediated activation of the receptor to reactivate the signal. Modification of Notch transcriptional activity by PTMs is critical for accurate Notch functionality (Andersson et al. 2011). For details considering the importance of oscillatory pattern expression of Notch target genes, please see the review of the literature section "1.5.1 Notch in differentiation in the CNS". As sumoylation is highly dynamic and reversible, this provides a mechanism for transcriptional switching.

1.7.4 Diverse effects of SUMO on gene expression

That stress-induced sumoylation of Notch repressed the expression of Notch target genes (Fig. 5D-K) is in accordance with previous data demonstrating that inhibition of global sumoylation increases Notch target gene expression (Licciardello et al. 2015). The cellular response to Notch activity is dose-dependent and the dose determines the balance between growth stimulative and suppressive effects (Mazzone et al. 2010). As already mentioned in the review of the literature, in section "1.4 Post-translational modifications of Notch", it has also been shown that NICD1 sumoylation is increased in mesenchymal stem cells (MSCs) with stabilized HIF-1α expression, leading to cell migration and invasiveness (Ciria et al. 2017). Although valuable discoveries, these two studies do not elucidate further aspects of the effect of sumoylation on the Notch pathway. If sumoylation of Notch is high, then Notch target gene expression will be lower. In most cases the proteins being synthesized from Notch target genes are gene inhibitory proteins that block the expression of genes, especially genes required for differentiation (Ratié et al. 2013).

A role and mechanism for Notch1 sumoylation has been proposed to occur in the endothelium. By targeting endothelial Notch signaling, sumoylation negatively regulates angiogenesis. Deletion of SENP1 enhances endothelial Notch sumoylation and prolongs Notch1 signaling, which suppresses VEGF receptor signaling and angiogenesis (Zhu et al. 2017). A recent study reported that SENP2 inhibits the Notch signaling pathway. However, the role of sumoylation itself was not studied (Chen et al. 2018).

As mentioned in the literature section "1.4.1 Sumoylation", the transcriptional activity of the CSL-interacting co-repressor protein KyoT2, on the Notch pathway is enhanced by its sumoylation. Although the study did not show KyoT2 sumoylation to decrease CSL-KyoT2 association, a sumoylation-deficient mutant KyoT2 resulted in repression of the KyoT2 transcriptional activity (Wang et al. 2007b). In contrast to KyoT2 sumoylation, MAML1 sumoylation decreases its transcriptional activity (Lindberg et al. 2010), which means that sumoylation may indirectly finetune activity of Notch target genes both in a repressive and enhancing manner. These data combined with the results from project (I) may raise

speculations if interlinking between sumoylation of NICD1, MAML1 and KyoT2 exist. For further details regarding SUMO network control, please see the literature section "2.4.5 Protein group sumoylation".

Repressed target gene expression in the presence of increased nuclear localization as an outcome of sumoylation has been demonstrated in other studies, for instance regarding the Forkhead transcription factor FOXL2 (Marongiu et al. 2010). In some cases sumoylation has been shown to lead to re-localization of the sumoylated protein within the nucleus, in combination with repressed gene activity (Oh et al. 2007). As explained later in the section "1.10.2 Sumoylated Notch recruits HDAC4 to repress gene expression", also recruitment of histone deacetylases (HDACs) by SUMO-conjugated NICD in the nucleaus, provides a mechanism for repressed transcriptional activity and is not contradictory with enhanced nuclear localization of sumoylated NICD (Fig. 3A-B, SI Fig. 3A-B) or with enhanced levels of NICD (Fig. 3E-F, SI Fig. 3C-E).

1.8 Notch sumoylation represses Hes5 expression in vivo in the CNS

In order to enhance the value and verify the results in experiments where mostly overexpressed proteins in cellular models were used, it was of importance to extend the findings also into a physiologically relevant context *in vivo*. We extended the study and analyzed the impact of heat shock and Notch sumoylation on Notch target gene expression in the developing chick CNS where Notch drives expression of Hes target genes which collectively function to inhibit differentiation of neuronal progenitor cells. Endogenous expression of the Notch downstream target genes Hes1 and Hes5 inhibits neural differentiation (Ohtsuka et al. 1999). Therefore, we electroporated chick embryos with vectors encoding either WT Notch-GFP-Flag or the sumoylation-deficient TSM Notch-GFP-Flag. We demonstrated that both WT Notch and the TSM induced expression of the target gene Hes5 in the control situation. However, as a consequence of heat shock, Hes5 expression was diminished in cells expressing Notch WT whereas it was still efficiently expressed in cells expressing the TSM (Fig. 6).

Although both Hes1 and Hes5 are known to inhibit neural differentiation (Ohtsuka et al. 1999), the effects in the form of differences in expression of Notch target genes, caused by WT Notch and the TSM in heat shocked conditions (Fig. 6) were not as definite or noticeable on Hes1 expression (SI Fig. 5), which is consistent with results demonstrating that the TSM did not in relation to WT Notch significantly enhance Hes1 expression in stressed cells *in vitro* (Fig. 5G). We also demonstrated that cell stress by bortezomib had an impact on Notch target gene Hes5 expression *in vivo* in the developing CNS of the chick embryo in a Notch-sumoylation dependent manner. In 50 μM bortezomib-stressed conditions, the expression of Hes5 was more repressed by WT Notch than by the TSM (SI Fig. 6). These experiments provide further evidence that Notch sumoylation occurs in a physiological setting.

As stated, Notch-mediated outcomes are heavily context-dependent and tissue-specific. In nervous tissue, Hes1 *activation inhibits* early differentiation of progenitor cells to neurons (Kobayashi and Kageyama 2014). This inhibitory effect by Hes1 on differentiation within one specific tissue cannot be assumed automatically to occur in other tissues and

circumstances as can be seen when compared for instance with muscle tissue. As also already previously mentioned in the literature section "1.3.3 The target genes Hes and Hey", Hes1 *activation promotes* myogenic differentiation. In turn, Hey1 activation inhibits myogenic differentiation (Rios et al. 2011; Nandagopal et al. 2018). It should however be noted that these kinds of snapshot analyses might not be sufficient to understand in detail the effect of pulsatile versus continuous NICD activity for muscle tissue differentiation. As an alternative or addition, live imaging of Hes and Hey activity could be used. Genetic inactivation of Hes1 combined with live imaging and lineage tracing has been used to demonstrate an endodermal requirement for Hes1 (Jørgensen et al. 2018). Live imaging has also been to used to show that CSL binds DNA very transiently during nonactive Notch signaling (Gomez-Lamarca et al. 2018). The CSL-DNA interactions are described more in detail the literature part of this thesis, section "1.3.2 Activation of Notch target genes".

Although a study has reported that global SUMO overexpression impairs neurogenesis (Juarez-Vicente et al. 2016) many transcription factors with roles in enhancing the expression of neuronal-specific genes, or in the downregulation of neuronal genes in nonneural cells, are regulated by being targets for SUMO conjugation. For instance, sumoylation of the transcription factor Pax-7 is needed for accurately steered neural crest development whereas sumoylation of the transcription factor BRAF35 mediates downregulation of neuronal genes in nonneural cells (Henley et al. 2014).

Progression and organization of development is strictly controlled with accurately timed stem cell proliferation and the differentiation of these into a variety of different cell types. Hes proteins regulate a variety of different events in development by, in many cases, blocking the expression of specific genes out of which many encode proteins that function as transcriptional activators that enhance cell differentiation. The genes encoding Hes1, Hes3 and Hes5 are all strongly expressed by neural stem cells. The inhibition of these genes causes premature differentiation of progenitor cells to neurons. Additionally, it has been shown that Hes1 expression dynamics is critical for proper timing of development, and for determining proper cell fate of embryonic stem cells, and also neural progenitor cells (Kobayashi and Kageyama 2014). Not much is known about target genes downstream of Notch and consequently about genes required for specification of neuronal development. Other Notch target genes during early chicken embryogenesis except the target genes studied in project (I), include Chromogranin A (Chga) and Contactin 2 (Cntn2) whose expressions are largely restricted to the nervous system (Ratié et al. 2014).

Cellular stress can in some cases also affect Notch signaling in a much more indirect, yet critical way, compared to cell stress-induced sumoylation of Notch. For instance, during metabolic stress, NF-kB upregulates the transcription of Notch ligands and receptors in the CNS leading to inhibition of neurogenesis (Bi and Kuang 2015). Low Notch activity upregulates the differentiation of neural stem cells to immediately supply neurons to repair nerve damage, while high Notch signaling activity facilitates quiescence and self-renewal in the form of proliferation of neural stem cells (Bi and Kuang 2015).

In project (I), neuronal sumoylation was indirectly studied with the cell stresses heat shock and bortezomib (Fig. 6, SI Fig. 5-6). However, these are not the only cell stresses that can cause enhanced neuronal sumoylation. In hippocampal neurons, SUMO1 and SUMO2/3 binding to their substrates is enhanced by OGD. Also SENP1 activity is enhanced during

OGD conditions pinpointing a complex crosstalk between sumoylation and desumoylation. Neuronal sumoylation in turn seems to have a neuroprotective role after OGD since overexpression of SENP1 leading to less sumoylation causes increased OGD-caused cell death of neurons (Cimarosti et al. 2012).

1.9 Sumoylation on the RAM domain does not block RAM-CSL interaction

Sumoylation of Notch repressed the expression of the studied human Notch target genes Hes1, Hey1 and Hey2 in HeLa cells during cell stress, whereas the TSM compared to WT Notch, increased the expression of the mentioned three target genes (Fig. 5D-K). Therefore, we sought to elucidate whether the mechanism underlying how stress-induced sumoylation of Notch decreased the expression activity of Hes1, Hey1 and Hey2 was a reduced interaction between sumoylated NICD and CSL. Since the RAM domain on NICD is the domain that NICD uses to bind directly to CSL and activate Notch target genes in canonical Notch signaling (Osipo et al. 2008), and since the RAM domain was verified to be an essential SUMO site (Fig- 5A), we postulated a potential mechanism where SUMO conjugated onto the RAM domain on NICD blocking the direct binding between NICD and CSL. Although earlier binding studies of RAM-CSL complexes have stated that the affinity of CSL for RAM is unaffected by the state of CSL being free in solution or bound to DNA (Friedmann et al. 2008), we did not observe consistent Notch-CSL interactions in free solution (data not shown). In order to secure their interaction, we crosslinked both Notch and CSL onto the DNA in a modified chromatin immunoprecipitation (ChIP) assay and studied this interaction in the presence and absence of SUMO during cellular stress (Fig. 7A). Our initial hypothesized mechanism of SUMO blocking RAM-CSL interaction turned out not to be the case as we were not able to detect a statistical difference in the WT NICD-CSL interaction in the presence and absence of overexpressed SUMO during heat shock (Fig. 7A). This finding was also in line with the three-dimensional model showing that K1780, K1781 and K1782 are easily accessible to sumoylation without affecting the RAM domain-CSL interaction or any other component within the NICD-CSL-MAML transcriptional complex (Fig 4, SI Fig. 4). Crosslinking proteins in ChIP may also pose different challenges as for instance the optimal crosslinking time varies depending on the proteins of interest. If the crosslinking time is too short the interaction between the proteins of interest and the chromatin may not take place, and if the crosslinking time is too long then there might occur unspecific binding to the chromatin.

These findings additionally supported each other. This led us searching for alternative mechanism(s) on how SUMO reduced the transcriptional activity of Notch target genes during cell stress.

1.10 Mechanism for SUMO-mediated attenuation of Notch target genes

Transcription factors recruit activating and repressing chromatin modifiers in order to regulate the landscape of chromatin and thus indirectly regulate the expression of genes. These genes are dependent on the correct steering of the signal in transduction pathways such as Notch. Generally, how signaling to chromatin occurs is not well known. Also, the mechanisms regulating Notch target gene expression in response to the physiological state

of the cell are mostly unknown. In the absence of NICD, CSL recruits transcriptional repressor proteins, but in the presence of NICD, transcriptional activators are recruited. Therefore, changes in the cellular environment require adaptable responses at the level of gene expression (Giaimo et al. 2017).

An important feature of sumoylation is the role of SUMO E3 ligases in SUMO conjugation to its target protein (Gong et al. 1999). When we screened various known proteins that are known to possess SUMO E3 ligase activity including PIAS1, PIAS2, PIAS3, PIAS4, HDAC4 and HDAC7, we did not observe any of them to specifically increase stress-induced sumoylation of Notch upon overexpression (data not shown). In parallel, we tested whether they could interact with sumoylated Notch, and whether they could interact with Notch during cell stress. HDAC4 specifically seemed to be such an interactor and interacted more potently with Notch in the presence of heat stress and SUMO (data not shown). That HDACs have been shown to possess capabilities to indirectly via sumoylation regulate transcriptional activities in the form of reduced gene expression (Garcia-Dominguez and Reyes 2009) led us to investigate more in detail the potential role of HDACs in the SUMO-mediated effect on Notch activity.

1.10.1 HDACs influence levels of gene expression

The chromatin itself is composed of both DNA and proteins. The genomic DNA in turn is wrapped around histones which are the main protein components of chromatin (Clocchiatti et al. 2011). Proper regulation of histone modifications is essential during development (Di Stefano et al. 2011). Histones are subjected to acetylation which is counterbalanced by deacetylation. These modifications affect the buildup of chromatin and also possess a regulatory role in gene expression including Notch target genes (Abend and Kehat 2015; Giaimo et al. 2017). The removal of acetyl groups from the N-terminal tails of histones is catalyzed by HDACs (Vaquero et al. 2007) which similar to Notch, have been shown to possess essential roles in differentiation (Clocchiatti et al. 2011).

Histone acetylation is often associated with enhanced gene expression whereas histone deacetylation generally correlates with repressed gene activity. A known mechanism by how sumoylation indirectly causes attenuation rather than promotion of gene expression is interaction between SUMO-conjugated proteins and HDACs (Garcia-Dominguez and Reyes 2009). HDACs are via deacetylating histones, and in some cases by HDACs recruiting and modifying additional repressors or corepressors, able to cause local alterations in the structure of the chromatin, resulting in turn in decreased transcription. HDAC4 which was identified around 20 years ago (Miska et al. 1999) has been shown to be recruited for instance by sumoylated LAP1, a member of the CEBP family of transcription factors. The HDAC4-LAP1 association in turn decreases the binding of HDAC4 on the cyclooxygenase 2 promoter, repressing its transcription (Wang et al. 2008).

Sumoylated MAML recruits HDAC7 (Lindberg et al. 2010), sumoylated p300 recruits HDAC6 (Girdwood et al. 2003) and sumoylated Elk-1 recruits HDCA2 (Yang and Sharrocks 2004), to repress transcriptional activity. Although independent of sumoylation, HDAC4 has also been shown to be recruited by transcription factors to repress interleukin-5 transcription (Han et al. 2006) and to associate with the myocyte enhancer factor MEF2

to repress MEF2 transcriptional activity, a function that requires the deacetylase domain of HDAC4 (Miska et al. 1999). HDAC4 is also recruited by the transcription factor YY1 to the HOXB13 promoter to repress transcriptional regulation of HOXB13 through modification of histone acetylation status (Ren et al. 2009). HDAC4 contains an N-terminal domain which is needed for the protein to interact with tissue-specific transcription factors, and to be recruited to their target genes (Parra 2015). HDAC4 has been shown to regulate gene expression during stem cell differentiation (Zhang et al. 2014) and tumorigenesis (Wilson et al. 2008; Chen et al. 2014).

1.10.2 Sumoylated Notch recruits HDAC4 to repress gene expression

HDAC4 knock down by siRNA increased Notch activity in the presence of heat shock (Fig. 7B, left panel), and much more so when SUMO was overexpressed during heat shock (Fig. 7B, right panel), which suggested that Notch-sumoylation mediated repression of Notch transcriptional activity required the presence of HDAC4. Following heat stress treatment, the presence of HDAC4 decreased the activity of WT Notch more than the sumoylation-deficient TSM, hinting that the mechanism through which HDAC4 dampened Notch activity, involved Notch sumoylation (Fig. 7C).

Since it seemed plausible that HDAC4 affected Notch activity via sumoylation, the next step was to verify whether NICD binds to HDAC4 and how cellular stress and SUMO overexpression affect the Notch-HDAC4 interaction. We observed an enhanced interaction between Notch and HDAC4 in heat shocked cells in which SUMO had been overexpressed (Fig. 7D). HDAC4 was bound more efficiently by WT Notch than by the TSM in response to heat shock and the presence of overexpressed SUMO, also supporting the notion that Notch sumoylation during cell stress enhances Notch-HDAC4 association (Fig. 7E). We also crosslinked Notch and HDAC4 onto the DNA in a modified ChIP assay to find out if they interact on the chromatin. The assay revealed the interaction to occur on the chromatin, although perhaps because of the messiness of the immunoblotting, a difference in the WT Notch-HDAC4 interaction and TSM Notch-HDAC4 interaction could not be observed (Fig. 7F).

Our results (Fig. 7B-F) implied that HDAC4 may have a decreasing effect on the expression of specific Notch target genes as a consequence of Notch sumoylation during cell stress. We decided to concentrate on the essential Notch target genes Hes1, Hey1 and Hey2. In the presence of heat stress and SUMO, silencing HDAC4 enhanced Notch target gene expression whereas overexpressing HDAC4 had an opposite impact. This experiment itself did not prove that the HDAC4-mediated dampening of Notch target gene expression occurred via Notch sumoylation since there was SUMO overexpressed in all samples, but these results (Fig. 7H-J) are in line with our earlier results (Fig. 7B-G). We included a schematic model (Fig. 8) to explain the likely mechanism by which stress-induced sumoylation of Notch mediates, by recruitment of HDAC4, the repression of specific Notch target genes. The mechanism will result in physiological changes within cellular levels as Notch is a crucial regulator of for instance stem cell differentiation and proliferation as described in project (II).

1.10.3 HDACs mediate SUMO-specific effects

Thousands of SUMO substrates have been identified and the number is growing as new SUMO target proteins are being discovered (Enserink 2017). HDAC4 is a universal histone deacetylase, but in addition, HDAC4 possesses SUMO E3 ligase activity (Garcia-Dominguez and Reyes 2009). There will therefore most likely be numerous genes affected by SUMO and HDAC4.

The importance of Notch regulation at this level is supported by a study demonstrating that site-specific methylation of NICD within the coactivator complex controls the amplitude and duration of the Notch response (Hein et al. 2015). The repressive role of HDAC4 on Hes1, Hey1 and Hey2 gene expression through Notch sumoylation by cell stress, is supported also by other reports. The mentioned event is in line for instance with studies showing that histones undergo deacetylation by oxidative stress (Niu et al. 2015) and osmotic stress (Magraner-Pardo et al. 2014), although the role of sumoylation might be unclear. Histones are subjected to several different PTMs including acetylation, methylation, ubiquitination and phosphorylation (Moshkin et al. 2009). These histone modifications which strongly influence gene transcriptional status, also regarding Notch target genes, can in some cases alter the structure of the chromatin and in some cases even recruit transcriptional factors to alter gene expression levels (Zhang et al. 2018b). Histones have been shown to undergo PTMs also as a consequence of different cellular stresses. These PTMs include histone methylation by oxidative stress (Chervona and Costa 2012; Niu et al. 2015) and hypoxic stress (Chervona and Costa 2012). Also phosphorylation of histone H3 is stress-inducible (Soloaga et al. 2003). Transcription factors and chromatin remodeling enzymes constitute one of the largest groups of proteins that are subjected to SUMO binding. Some groups of transcription factors and chromatin modifiers are sumoylated during cell stress whereas some are desumoylated (Enserink 2017). In a proteomics approach, 20 upregulated and 33 downregulated chromatin modifier SUMO2-substrates were identified in response to DNA damage (Hendriks et al. 2015). Additionally, HDAC1 itself has been shown to be sumoylated during myogenesis (Joung et al. 2018), and high mobility group A2 (HMGA2) was the first nonhistone chromosomal architectural protein reported to be SUMO-conjugated (Cao et al. 2008).

Notch transcriptional activity is dependent on the chromatin status of its target genes (Sancho et al. 2015), which based on project (I), seemed to be affected by HDAC4 which in turn was recruited by sumoylated Notch during cell stress. SUMO2 has been displayed to accumulate, in response to heat shock, at active DNA-regulatory elements which function as binding sites for large protein complexes and are mostly associated with active genes. The same study suggested that SUMO2-conjugation functions as an acute cell stress response mechanism that is required for maintaining stability of protein complexes that are involved in gene expression (Seifert et al. 2015), which may apply also for the NICD-CSL-MAML transcriptional complex.

Potentially sumoylation of many components of the regulatory circuit may be needed to elicit a physiological response. Sumoylation suppresses expression of Notch target genes by recruiting HDAC4 to the transcriptional complex and thereby provides a dynamic switch in transcriptional activity. The importance of HDACs in coordination of Notch signaling activities in differentiation in the peripheral nervous system has been demonstrated. The

Zeb2 (also known as SIP1) transcription factor is a critical intrinsic timer that controls the onset of Schwann cell differentiation by recruiting histone deacetylases HDAC 1 and 2 (HDAC1/2) to suppress the Notch-Hey2 signaling axis (Wu et al. 2016).

1.10.4 Potential future studies for HDAC4 in the context of Notch sumoylation

Project (I) strongly suggested that sumoylation of Notch constitutes a new mechanism for dynamic control of Notch transcriptional activity through the recruitment of HDACs to the transcriptional complex. The work in project (I) opens up questions, which warrants further studies. To study in more detail the mechanistic role of HDAC4 in the repression of Notch target genes at a chromatin level as a consequence of stress-inducible Notch sumoylation, would be of value for understanding the role of SUMO in the Notch pathway. Addressing this question would further outline molecular mechanisms underlying Notch sumoylation and its biological consequences.

Currently, although strong indications in project (I), we did not specifically verify that HDAC4 recruitment by sumoylated Notch leads to 1) deacetylation of histones at the promoter of Notch target genes, or 2) deacetylation or recruitment of other transcriptional regulators to the transcriptional complex, which are mechanisms through which HDACs are known to mediate transcriptional repression (Kim et al. 2007; Guarani et al. 2011; Popko-Scibor et al. 2011; Palermo et al. 2012; Jin et al. 2017). However, we so far demonstrated a variety of results strongly indicating a role for HDAC4 in repressing Notch target gene expression via stress-induced sumoylation of Notch. We showed that the mechanism by which stress-induced sumoylation represses the expression of Notch target genes was not a reduced Notch-CSL interaction (Fig. 7A), that sumoylated Notch interacted more potently with HDAC4 (Fig. 7D) and that the Notch-HDAC4 interaction occurred on the chromatin (Fig. 7F). The last notion (Fig. 7F) was supported by data demonstrating that WT Notch1 interacted more efficiently with HDAC4 than the sumoylation deficient TSM (Fig. 7E). In addition, silencing of HDAC4 enhanced Notch activity (Fig. 7B) and Notch target gene expression (Fig. 7H, 7I, 7J).

Whether HDAC4 is bound to the Notch transcriptional complex and whether Notch sumoylation increases the binding of HDAC4 to the Notch transcriptional complex could be studied by performing IP using Notch and CSL antibodies, followed by immunoblotting against HDAC4. In this case, heat shocked cells could be compared to non-heat shocked cells. By using the sumoylation-deficient TSM as a negative control it could be verified that the interaction of HDAC4 to the Notch transcriptional complex during heat shock occurs as a result of Notch sumoylation and not something else. The strongest interaction would expectedly occur in heat shocked cells that have been overexpressed with SUMO and WT Notch.

However, there are still main questions to be addressed. The first essential question is **does the Notch-HDAC4 interaction occur on a Notch target gene promoter?** The modified ChIP assay experiment (Fig. 7F) could be extended and could potentially verify that cell stress induced Notch sumoylation increases WT Notch-HDAC4 interaction on the chromatin, but the method cannot verify the interaction to occur specifically on a Notch target gene promoter. The question of Notch-HDAC4 interaction on Notch target gene

promoter still remains to be addressed more thoroughly as part of further studies. This could be studied by doing ChIP-RT-PCR, by first using Notch, CSL and HDAC4 antibodies after which RT-PCR would be performed by using primers that multiply a promoter region of some Notch target gene.

The second essential question is a more detailed follow-up question of the first question: **does Notch sumoylation increase HDAC4 presence specifically on a Notch target gene promoter?** The mentioned ChIP-RT-PCR could be used to address this question by studying the multipilication of the promoter region in the presence and absence of overexpressed SUMO, and in the presence and absence of heat shock. However, this question can be studied also separately from ChIP-RT-PCR. Also, traditional ChIP could be used to determine if stress-induced sumoylation of Notch enhances the occupancy of HDAC4 on a Notch target gene promoter. An alternative newer method include cleavage under targets and release using nuclease (CUT&RUN)-sequencing which can be used to identify the binding sites of DNA-associated proteins. In CUT&RUN, micrococcal nuclease targets antibody for controlled cleavage releasing the wanted protein-DNA complexes into the supernatant, followed by DNA sequencing. Whereas the more traditional ChIP method fragments and solubilizes total chromatin, CUT&RUN is done *in situ*, which allows for quantitative high-resolution chromatin mapping and probing of the chromatin environment of interest (Skene and Henikoff 2017).

It is a potential event for instance that a SUMO-conjugated NICD recruits HDAC4 directly to a Notch target gene promoter where HDAC4 deacetylates histones. These deacetylated histones would thereby be more able to bind to DNA and thus restrict more efficiently access of transcription factors to the DNA and consequently cause repression of Notch target gene expression. If this is accurate then the TSM starts another chain reaction: no Notch sumoylation, no HDAC4 recruitment, histones are staying acetylated and bind poorly to DNA allowing NICD to more efficiently bind to the promoter causing higher gene activity. Since sumoylation regulated the abundance of NICD (Fig. 3E-F, SI Fig. 3C-E) then ChIP would be an appealing method to determine also whether TSM or +/- SUMO during heat stress affects the occupancy of NICD on a target gene. Stated differently, it could also be studied if the transcriptional effects were due to changes in the amount of NICD that binds to a target gene.

Since Notch and HDAC4 interacted on the chromatin (Fig. 7F) the third essential question is **does Notch sumoylation lead to more deacetylation of histones on a Notch target gene promoter?** Therefore, it would be of interest to examine how histone acetylation at Notch promoters, as a readout of HDAC4 activity, might be influenced by Notch sumoylation. For this ChIP-RT-PCR could be performed, by first using antibodies that recognize acetylated histone H4, and other antibodies that recognize total histone H4, after which RT-PCR would be performed by using primers that multiply the promoter region of some Notch target gene. This ChIP-RT-PCR could be performed by using WT Notch vs the TSM, presence and absence of SUMO, and presence and absence of heat shock.

Perhaps the most likely mechanism by how HDAC4 as a result of stress-induced Notch sumoylation decreases Notch target gene expression is either HDAC4 deacetylating histones or other proteins in the Notch-CSL transcriptional complex on chromatin at a promoter of Notch target genes. In project (I) we demonstrated sumoylation-induced

recruitment of HDAC4 to the Notch transcriptional complex on DNA, and demonstrated that SUMO and HDAC4 affects Notch target gene expression and that this requires site-specific sumoylation of Notch. Further studies to elucidate the mechanistic role of HDAC4 in the repression of Notch target genes during cell stress would hopefully be of interest for researchers focused on transcriptional regulation.

1.11 Conclusions of the interaction between Notch and SUMO

The Notch signaling pathway is a key regulator of tissue development. Notch signaling output is diverse and dependent on cell context, emphasizing the necessity of cells to modify Notch target gene expression in response to physiological changes (Mohtashami et al. 2010). In project (I), we uncovered a new regulatory mechanism of Notch and demonstrated that NICD is modified by SUMO in the nucleus (Fig. 3C-D), and that sumoylation is enhanced during cell stress (Fig. 2C-D, SI Fig. 1A, SI Fig. 2C, SI Fig. 3C). Our data revealed that stress-inducible sumoylation regulated Notch transcriptional activity (Fig. 5D-K, Fig. 7H-J). More mechanistically, in project (I) our data supported the events that Notch1 is sumoylated on the NICD RAM domain within the Notch transcriptional complex upon heat stress, which increases the levels of NICD in the nucleus, and increases the NICD-HDAC4 interaction. The data suggests that this facilitates the recruitment of HDAC4 to the transcriptional complex within the DNA, to the promoter region of Notch target genes to repress their expression.

Transduction of the Notch signaling pathway is quite simple compared to many other signaling pathways, partly because the activation and amplification of the Notch signal is not dependent on secondary messengers. Simultaneously Notch is very pleiotrophic regarding the ouput of activity as highlighted by its tissue-specific and context-dependent outcomes. The evolutionarily conserved Notch signaling pathway regulates development and renewal of most tissues and is deregulated in a variety of cancers. Many developmental events require oscillating and dynamic expression of Notch target genes, mediated in part by inherent feedback loops and chromatin modifications. For accurate steering of stem cell function during development, and for cellular responses to changes in the physiological environment, it is crucial that expression of Notch target genes is regulated at an oscillatory and dynamic level. The mechanisms underlying the dynamic regulation of Notch target genes, and Notch signaling diversity are inadequately known. In order to more thoroughly comprehend the role of Notch in development and cell fate it is therefore essential to characterize the regulatory factors of Notch and expression of its target genes.

Project (I) brought forward new molecular information on how cellular stress and SUMO in concert modify the activity of NICD1 and how this PTM of Notch regulates Notch downstream functions. Altogether, these data suggest a model whereby sumoylation provides a mechanism for fine-tuning Notch-mediated cell functions in response to changes in the physiological state. Many nuclear Notch-interacting proteins have been discovered within the last decade, many of which have been shown to be key regulators of Notch transcriptional activity and oncogenic function (Figure 29) (Yatim et al. 2012).

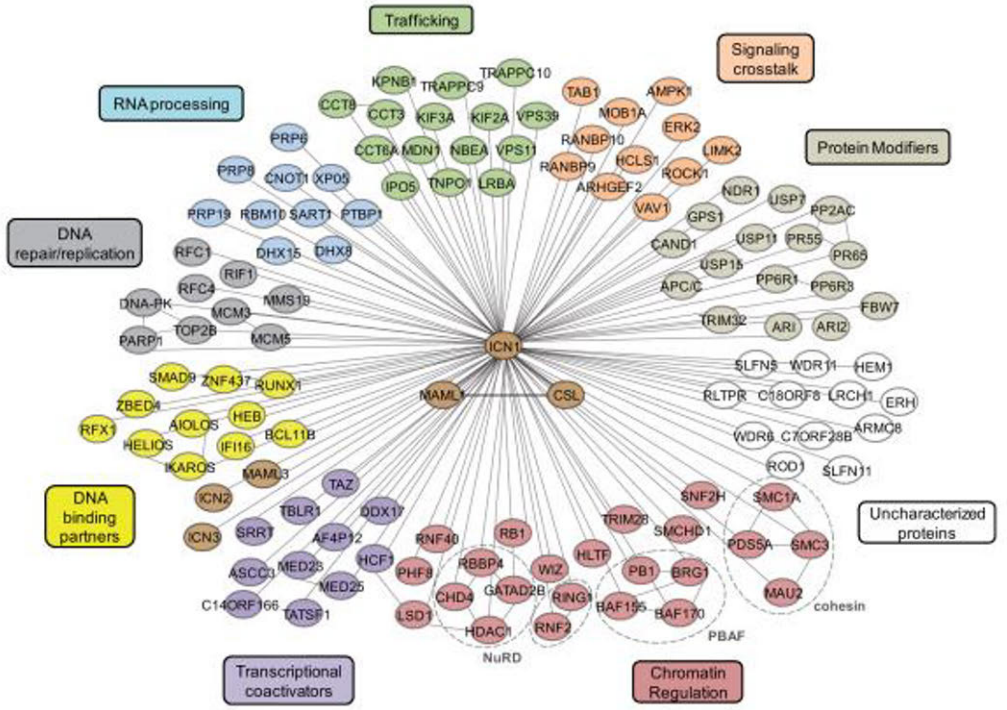

Figure 29. Interaction network of NICD1-associated proteins identified by masspectrometry. Figure is modified from (Yatim et al. 2012).

Project (I) is the first article that addresses and characterizes Notch sumoylation in detail. It is not unconceivable that a greater number of Notch-related sumoylation articles will be published within the upcoming years as project (I) has opened and discovered this recently unexplored area of Notch activity regulation.

2. Keratins regulate colonic epithelial cell differentiation through the Notch1 signaling pathway

The data discussed in chapter 2 of the results and discussion section is part of aim 2 and presented in the publication Lähdeniemi, Misiorek*, Antila* et al, Keratins regulate colonic epithelial cell differentiation through the Notch1 signaling pathway. *Cell Death Differ*. 24:984-996, 2017.

Notch controls tissue homeostasis in the colon and exploring how Notch is regulated in colonic epithelial cells is a key aim of this thesis work. Project (II) focuses on the effect of our discovered Notch-keratin interaction on the Notch signaling pathway and consequently on the proliferation and differentiation of colonic epithelial cells.

The intestine undergoes constant rapid regeneration to maintain tissue homeostais, which is being carried out in a relatively simple repetitive manner and therefore the intestinal epithelium is nowadays a widely used model in stem cell research (Clevers 2013). Most research on simple epithelial keratins (SEKs: K7-K8, K18-K20) has been performed in mammalian systems such as mouse models, cell cultures and in human samples. This is partly because more simple organisms including *Drosophila melanogaster* and bacteria lack endogenous SEKs, and partly because of the relevance of keratin isoform variants in the development of human diseases (Strnad et al. 2016).

In project (II) we utilized $K8^{+/+}$, $K8^{+/-}$ and $K8^{-/-}$ FVB/N mouse strains, and *in vitro* cell cultures. The heterozygote $K8^{+/-}$ mouse strain has only half of the K8 amount compared to its corresponding homozygote $K8^{+/+}$ strain, resulting in decreased levels of also all the other colonic SEKs K7, K18-K20 within the $K8^{+/-}$ strain. In relation to the $K8^{+/+}$ and $K8^{-/-}$, the $K8^{+/-}$ mice carry an intermediate and mild phenotype which is demonstrated by $K8^{+/-}$ mice developing colonic epithelial hyperproliferation and defects in ion transport similarly as in $K8^{-/-}$ mice. However, in contrast to $K8^{-/-}$ mice, $K8^{+/-}$ mice do not develop colonic inflammation (Asghar et al. 2015).

Since Notch and keratins mediate widely essential functions in the colon, the newly discovered interaction of these proteins will significantly help in understanding colonic homeostasis in more detail. If not stated otherwise, the results in project (II) concerning Notch and more specifically NICD refer to the full length receptor protein isoforms, Notch1, and the intracellular domain, NICD1, respectively.

2.1 K8 interacts with Notch

Notch is known to be an important regulator of proliferation and differentiation of colonic epithelial stem cells (Naito et al. 2017) whereas K8 is the most common type II keratin in the intestine (Zhou et al. 2003). In the colonic crypts, $K8^{-/-}$ mice display colonic epithelial hyperproliferation (Habtezion et al. 2011). This indicated a function for keratins in epithelial tissue differentiation and concurrently a link between keratins and Notch.

In order to explore if there is a direct Notch-keratin interaction, we analyzed protein interactions by immunoprecipitation of K8/K18 *in vivo* and detected NICD both in murine distal colon (DC) and proximal colon (PC) (Fig. 1A, SI Fig. 1A). The colon is divided into proximal colon (cecum, ascending colon, hepatic flexure, transverse colon) and the distal colon (descending colon, sigmoid colon, rectum) (Lin et al. 2016).

The nature of keratins being obligate heteropolymers makes it necessary to express an equal amount of type I and type II keratin DNA whenever overexpression of SEKs is used (Strnad et al. 2016). The Notch-keratin interaction was additionally shown *in vitro*, where Notch was immunoprecipitated, and K8 and K18 detected in the precipitate of MEFvim$^{-/-}$ mouse fibroblast cells lacking the IF vimentin (Fig. 1B, SI Fig. 1B). The Notch-keratin interaction was also seen in HEK 293 FLN cells where endogenous K18 was immunoprecipitated and Notch detected in the precipitate (SI Fig 1C). In human fetal kidney, keratins and vimentin are often found in the same cell whereas in adults these two proteins are only rarely co-expressed in the same cell (Moll et al. 1991).

K8/K18 seemed to interact with all three Notch forms: with NICD (Fig. 1A-B, SI Fig. 1A), and with ΔEN (SI Fig. 1B), and also with FLN (SI Fig. 1B-C). All these three forms of Notch contain the intracellular domain. This suggests that also ΔEN and FLN may interact with keratins via their NICD domain although this was not experimentally addressed. It is also possible that keratins interact with ΔEN and FLN at some site(s) outside the intracellular domain.

Phosphorylation of K8 S74 is one of the most important PTMs on K8. This particular PTM is known to have a role in mitosis, apoptosis and interestingly also in cell stress (Snider and Omary 2014). For further elucidation of the relevance and role of K8 S74 phosphorylation in biological functions, please see the literature section "3.4.2 Keratins as cellular stress protectors". We tested whether this essential PTM impacts on how K8 interacts with and regulates the Notch signaling pathway. Based on immunoprecipitation and immunoblotting experiments, K8 S74A bound Notch in a similar manner as K8 WT (Fig. 1B) indicating that K8 S74 phosphorylation does not affect Notch-K8 interaction.

2.2 K8 co-localizes with Notch in vitro

As K8 binds to all Notch constructs (Fig. 1A-B, SI Fig. 1A-C) we used immunostainings and proximity ligation assays (PLA) to test if Notch and K8 interact at the cell membrane or in the cytoplasm. FLN and ΔEN reside at the cell membrane, and NICD in the cytoplasm and in the nucleus (Naito et al. 2017). Because of the predominantly cytoplasmic nature of keratins (Hobbs et al. 2016), a nuclear K8-Notch interaction would be expected to be difficult to detect.

We showed by PLA (Fig. 1D) and immunofluerescence staining (SI Fig. 2A) that K8 and Notch co-localize in the cytoplasm and the cell membrane in Caco-2 human colorectal cancer cells. The co-localization was also demonstrated in MCF7 breast cancer cells (SI Fig. 2B). The co-localization in the cytoplasm as well as at the cell membrane indicated that K8 interacts both with the S3 cleaved cytoplasmic NICD, and with the membrane bound FLN Notch or the membrane-tethered ΔEN, and combined with the immunoblotting

results (Fig. 1A-B, SI Fig. 1A-C) it seemed that K8 interacted with all forms of Notch. Although perhaps unlikely, the outcome of K8-FLN, K8-ΔEN and K8-NICD interactions may be different making keratins potentially regulators of different aspects of the Notch signaling pathway at different levels of the pathway. For thoughts on direct mechanistic outcomes of these bindings on the Notch signaling mechanism, please see section "2.9 Conclusions of the interaction between Notch and keratins". Previously Notch2 has been shown to co-localize with K8 in the ureteric bud (UB) of murine kidney at E17.5 (Liu et al. 2013).

Intermediate filament proteins including keratins do not have any specific centers for assembly into filaments. Keratins can form filaments throughout the cytoplasm (Haines and Lane 2012). Keratins were formerly referred to as cytokeratins because it was believed that they only exist in the the cytoplasm, but nowadays evidence point to the fact that keratins function also in the nucleus (Hobbs et al. 2016). K17 has been detected in the nucleus of cervical cancer epithelial cells affecting proliferation and gene expression (Escobar-Hoyos et al. 2015). Additionally, K8 and K18 along with K7 and K17 have been identified in a screen for nuclear matrix proteins that shuttle back and forth between the cytoplasm and nucleus in cultured HeLa cells (Kumeta et al. 2013). We did however not detect Notch1 and K8 co-localization in the nucleus (Fig. 1D, SI Fig 2A-B). Another study also highlights the effect of keratins on Notch, by linking K19 and Notch2. The project showed that K19 KO mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) deteriorated Notch2 signaling in biliary epithelial cells (Chen et al. 2015).

2.3 Keratins increase Notch levels and target gene expression in vitro

Interaction and co-localization of Notch and K8 (Fig. 1, SI Fig. 1, SI Fig. 2A-B) suggested that a functional purpose for the Notch-K8 binding exist and consequently led us to investigate the role of K8 in the regulation of Notch1. K8/K18 did not affect the levels of endogenous FLN or NICD in mouse embryonic fibroblast cells lacking vimentin intermediate filaments (MEF $vim^{-/-}$) (Fig. 2A), but overexpression of keratins increased NICD levels (Fig. 2C, 2F). Removal of phosphorylation site S74 on K8 did not have an effect on NICD levels (Fig. 2C), consistent with the phosphorylation site not affecting K8-Notch binding (Fig. 1B). Enhanced NICD levels were also detected in a keratin-dose dependent manner (Fig. 2E). The effect of keratins enhancing NICD levels seemed to stem mostly from the K8 isoform specifically since the expression of both K8/K18 and K8/K19 resulted in a similar increase of NICD levels (Fig. 2F).

Overexpression of K8/K18 WT increased Hey1 and Hey2 mRNA levels (Fig. 2G). Overexpressed K8 S74A in turn showed less expression of Notch target genes, compared to K8 WT (Fig. 2G). K8 S74 phosphorylation thereby seemed to increase Notch target gene expression (Fig. 2G). Compared to K8 WT, K8 S74 phosphorylation did not affect NICD levels upon proteasome inhibition (Fig. 2H). WT K8 overexpression did not, at least significantly, result in higher NICD levels upon proteasome inhibition, which indicated that keratins do not significantly affect the NICD degradation rate (Fig. 2H). This seemed to be consistent with results that showed that overexpression of keratins did not affect the rate at which NICD levels decreased after cells had been treated with the protein translation inhibitor cycloheximide (CHX) for different time periods (SI Fig. 3A-C). Consistent with

previous results showing that WT K8/K18 enhance NICD levels (Fig. 2C, E, F), NICD levels were higher at all time points of (CHX) treatment when keratins had been overexpressed (SI Fig. 3A-B). FLN degradation rate was also independent of the presence of K8 (SI Fig. 3D), and high salt extractions from $K8^{+/+}$, $K8^{+/-}$ and $K8^{-/-}$ colon showed that FLN was present in the high salt cytoskeleton (Fig. 2J).

2.4 Notch levels and target gene expression are decreased in the $K8^{-/-}$ colon

To gain more significance and more supportive results for the previously mentioned intestinal epithelial *in vitro* results, it was essential to study the effect of K8 on Notch in a more natural setting in the form of colonic *in vivo* environments. By using colonic epithelium from different mice strains of FVB/N, we observed a decrease of both FLN and NICD levels in the $K8^{-/-}$ colon vs $K8^{+/+}$ colon (Fig. 3A), which was consistent with results showing that overexpression of WT K8/K18 enhance NICD levels (Fig. 2C, E, F, SI Fig. 3A-B) *in vitro*. It is important to note that once K8 has been deleted, it additionally leads to the deletion of its heterodimeric partner protein K18 (Fuchs and Weber 1994; van Es et al. 2005). Further, immunohistochemical staining confirmed that NICD levels were decreased throughout the $K8^{-/-}$ crypt (Fig. 3D).

Because it has been shown that luminal bacteria within the colon can affect expression of Notch target genes (Becker et al. 2013), we wanted to eliminate the possibility that bacteria could cause differences in Notch protein levels and/or target gene expression levels when comparing $K8^{+/+}$ and $K8^{-/-}$ colon. This was important as $K8^{-/-}$ mice suffer from intestinal inflammation (Baribault et al. 1994) which can be caused by colonic luminal bacteria. Thus, compared to K8 WT mice, $K8^{-/-}$ mice may potentially have more intestinal bacteria which are capable of activating Notch target genes. Thereby we decreased/altered the balance of colonic bacteria by antiobiotic treatment, and observed a similar decrease of FLN levels in the $K8^{-/-}$ colon compared to the $K8^{+/+}$ colon in mice treated with antibiotics (Fig. 3E-F) as untreated mice (Fig. 3A-B) verifying that colonic bacteria did not regulate Notch levels. Alternatively, the effect of antiobiotic treated $K8^{-/-}$ mice versus untreated $K8^{-/-}$ mice on Notch target gene expression could have been studied. In this hypothetical experiment if bacteria did not affect Notch target gene expression then the expression effect of both the antibiotic treated $K8^{-/-}$ mice and the untreated $K8^{-/-}$ mice on Notch target genes should be the same.

Elimination of K8 did not affect Notch mRNA levels (Fig. 3G), which indicates that the effect that keratins display on the Notch signaling pathway occurs, concurrent with all earlier results presented within project (II), specifically on the Notch protein. The expression of Notch target genes Hey1 and Hey2 was repressed in the absence of K8 (Fig. 3H), which was consistent with K8 overexpression leading to enhanced Notch target gene expression *in vitro* (Fig. 2G).

2.5 Notch levels and target gene expression are decreased in CRISPR/Cas9 K8$^{-/-}$ colon cells and rescued by K8/K18 re-expression

In vitro in MEF vim$^{-/-}$ cells, we did not detect a difference in endogenous FLN or NICD levels in the absence or presence of specifically overexpressed K8/K18 (Fig.2A). This might be related to an issue of detecting endogenous basal Notch levels *in vitro* specifically in this setting. As an alternative model, we used CRISPR/Cas9 K8$^{-/-}$ human colorectal cancer Caco-2 cells, and observed that compared to K8$^{+/+}$ cells, both FLN and NICD endogenous levels were decreased (Fig. 4A) and expression of the Notch target gene Hey1 was reduced (Fig. 4D). In addition, also immunofluorescence staining demonstrated loss of Notch in CRISPR/Cas9 K8$^{-/-}$ Caco-2 cells (Fig. 4Ed). Consistently, the presence of Notch could be partially regained when K8/K18 (Fig.4Eg) or K8/K18 S74A (Fig. 4Ej) were overexpressed in K8$^{-/-}$ cells.

Immunostaining with an antibody against Hey1 revealed that the presence of this protein was reduced in CRISPR/Cas9 K8$^{-/-}$ Caco-2 cells (Fig. 5D) in a similar manner as when immunostained for Notch (Fig. 4Ed). Hey1 levels were also regained by K8/K18 expression (Fig. 5G). Hey1 levels were not regained by expression of K8 S74A/K18 (Fig. 5J). The fact that K8 S74A, compared to K8 WT, reduced Hey1 protein abundance (Fig. 5J) was in line with repressed Hey1 gene expression with K8 S74A, compared to K8 WT (Fig. 2G). This suggests a role for K8 S74 phosphorylation in enhancing Hey1 mRNA levels.

2.6 Effect of K8 S74 phosphorylation on Notch signaling

Since the K8 S74 phosphorylation mediates essential functions during mitosis, apoptosis and cell stress (Snider and Omary 2014), and regulates keratin filament reorganization (Ku et al. 2002), it was important to find out if the phosphorylation has a role in how K8 affects Notch signaling.

The S74 phosphorylation on K8 did not affect binding of keratins to Notch (Fig. 1B), Notch levels (Fig. 2C, 2H, SI Fig. 3A-B, Fig. 4) or NICD degradation rate/turnover (Fig. 2H, SI Fig. 3A-D), but it did decrease expression of Notch target genes Hey1 and Hey2. Expression of both target genes was higher when K8 WT was overexpressed compared to when K8 S74A was overexpressed (Fig. 2G), which is in line with K8 deletion downregulating Hey1 levels in CRISPR/Cas9 Caco-2 cells in which Hey1 levels were not rescued as efficiently by K8 S74A as by K8 WT (Fig 5G, 5J). These results indicate that K8 S74 phosphorylation enhances Notch target gene expression.

It has been shown that K8 S74 phosphorylation makes keratin filaments more soluble (Snider and Omary 2014). Considering this, it is possible that K8 S74 phosphorylation releases NICD from keratin filaments thereby enhancing NICD nuclear translocation, which would further lead to elevated expression of Notch target genes. However, we observed no detectable difference in the binding of K8 WT and K8 S74A to Notch (Fig. 1B). This in turn may be a result of sensitivity of the method, or a fine-tuning mechanism.

As mentioned in the literature part, section "3.4.2 Keratins as cellular stress protectors" K8 S74 phosphorylation by the kinase p38 is the most prevalent PTM on keratins, and occurs in organs and cultured cells during cell stress, mitosis and apoptosis, and regulates keratin filament reorganization (Ku et al. 2002). Since K8 S74 is phosphorylated upon cellular stress by p38 (Ku et al. 2002) and c-Jun N-terminal kinase (JNK) (He et al. 2002; Ho and Artavanis-Tsakonas 2016) thereby possibly releasing Notch from keratin filaments leading to enhanced nuclear Notch localization and further to enhanced Notch target gene expression, this could possibly provide, at least partly, a mechanism on how heat shock itself without recovery time enhances Notch target gene expression (SI Fig, 2B, study I). The enhanced expression of Notch target genes by heat shock itself (SI Fig. 2B, study I) may perhaps also result from heat shock reorganizing keratins and increasing the amount of keratins thorugh increased expression of keratins, which would mean that there would be more keratins to enhance Notch target gene expression. These potential events are supported by K8, K18 and vimentin being the main intermediate filament proteins expressed by monkey kidney COS7 cells (Karashima and Watt 2002) used in SI Fig. 2B, project (I).

2.7 K8 KO leads colonic cell differentiation towards a secretory cell fate

Homeostasis in the intestinal colonic epithelial tissue requires a pure balance of self-renewal between stem cells residing in the colonic crypts, and differentiation of the stem cells towards specialized cells (Weichselbaum and Klein 2018). For detailed introduction of the roles of enterocytes, goblet cells and enteroendocrine cells in the intestinal epithelial tissue, please see the literature section "3.1.1 Proliferation and differentiation of colonic epithelial stem cells and progenitor cells".

Prior to the initiation of project (II) it was unknown whether keratins are involved in the regulation of differentiation of epithelial cells in the colon. In addition to K8 and K18 being the most abundant keratin isoforms in epithelial cells, they also constitute the most common keratin proteins being synthesized early in development (Strnad et al. 2016). Notch in turn has been shown to have a very strong regulatory role in colonic epithelial cell differentiation (Roulis and Flavell 2016). It has been shown earlier that functional active Notch signaling will cause TA progenitor cells to differentiate predominantly into enterocytes within the absorptive cell lineage, whereas inhibited Notch signaling in turn will cause all TA cells to specialize towards the secretory cell lineage consisting of goblet cells and enteroendocrine cells (Clevers 2013). Therefore, it was of interest to analyze if K8, via directly regulating Notch signaling activity, has an indirect impact on the differentiation of TA cells towards some specific cell lineage fate. By utilizing the actin-binding villin protein that participates in the assembly of the microvillus bundle, as a marker for enterocyte cell types (Robine et al. 1997), a robust decrease of villin in $K8^{-/-}$ colon was observed, most likely representing reduced amounts of enterocytes (Fig. 6A). Concurrently, in $K8^{-/-}$ mice, the mRNA levels of the enterocyte secreted carbonic anhydrase (CA2) enzyme which catalyzes the reversible hydration of carbon dioxide (Chantret et al. 1988; Robine et al. 1997; Bekku et al. 1998) was also dampened (Fig. 6C). The goblet cell marker mucus (Garg et al. 2007) was accordingly increased in $K8^{-/-}$ mice (Fig. 6D, SI Fig. 4), although perhaps because of failed terminal differentiation of TA cells, the mRNA levels of the proteins Mucin1/2 secreted by goblet cells were lower than those secreted by $K8^{+/+}$ (Fig. 6E). Failed terminal

differentiation has been detected for instance as reduced amounts of goblet cells through loss of the Wnt-responsive, Ets-domain transcription factor Spdef (Gregorieff et al. 2009).

Regarding the effect of keratins on enteroendocrine cell amounts, the levels of the enteroendocrine cell marker synaptophysin membrane glycoprotein (Gunawardene et al. 2011) was increased in $K8^{-/-}$ mice (Fig. 6F). Also other markers are available for enteroendocrine cells, such as INSL5 (Thanasupawat et al. 2013). The increased amounts of enteroendocrine cells in the absence of K8 was also presented with immunofluorescence staining. The increased amount of synaptophysin-positive enteroendocrine cells was detected mainly in the crypt bottoms of $K8^{-/-}$ mice colon (Fig 6H). Enteroendocrine cells are localized as individual cells throughout the mucosa and represent only 1% of the cells lining the intestinal lumen (Gunawardene et al. 2011), which might partly explain why inhibition of Notch signaling in the intestinal epithelium by deletion of CSL or by γ-secretase inhibitors leads mostly to increased amount of goblet cells specifically and not enteroendocrine cells (van der Flier and Clevers 2009), still taking into account that these two specialized forms of cells are members of the same secretory cell lineage.

The depletion of K8 seems to cause TA cells to differentiate into goblet cells and enteroendocrine cells in the secretory cell lineage (Fig 6). This occurs most likely from K8 loss causing loss of Notch that leads to a consequent reduction of mainly the Hes1 (also Hes3 and Hes5) gene expression whose protein product Hes1 is not present to block the expression of the Math1/Atoh1 gene. Therefore, the Math1/Atoh1 gene which functions as the gatekeeper of entry of TA cells into the secretory cell lineage can freely allow the differentiation of TA cells into goblet cells and enteroendocrine cells. Most likely overexpression of K8 would via the Notch signaling pathway lead to TA cells differentiating much more into the absorptive cell lineage consisting of only enterocytes.

It has been shown that when Notch is acutely inhibited all proliferative TA progenitor cells differentiate into goblet cells and not enteroendocrine cells or tuft cells, which would indicate that within the secretory cell lineage it is specifically goblet cells that represent the default cell fate of former TA cells (Clevers 2013). This is also in line with goblet cells comprising 16% of the cells in descending colon (van der Flier and Clevers 2009) whereas enteroendocrine cells in turn constitute only 1% of the cells in colonic crypts (Sancho et al. 2015). In comparison, enterocytes of the absorptive cell lineage make up over 80% of all intestinal epithelial cells (van der Flier and Clevers 2009). TA cells did not differentiate only into goblet cells, but also into enteroendocrine cells, in the absence of K8 (Fig. 6).

All in all, the decreased amounts of villin representing enterocytes (Fig. 6A), the increased amounts of acidic mucus stained by alcian blue staining representing goblet cells (Fig. 6D), and the increased amounts of synaptophysin representing enteroendocrine cells (Fig, 6F, H) combined strongly support a dose-dependet effect of K8, by altering Notch signaling activity, on lineage specification of TA cells.

2.8 K8 KO leads to a widened proliferative zone and more TA cells

In addition to investigating the effect of keratins steering the differentiation of TA cells into specific cell lineages (Fig. 6, SI Fig. 4) we also examined if keratins have an impact on the actual amount of TA cells. We used the protein phosphohistone H3 (PHH3) as a marker of TA cells (Colman et al. 2006) to examine this. The levels of PHH3 were significantly higher in the colon of $K8^{-/-}$ mice, compared to WT mice, which indicated that the amount of TA cells was increased as a consequence of the absence of K8 (Fig. 7A).

In the stem cell niche, Notch activity maintains the intestinal stem cell pool by promoting symmetric proliferation of stem cells into two daughter stem cells, and thereby prevents the formation of TA cells from intestinal stem cells (Sancho et al. 2015). Loss of K8 (Fig. 7A) thereby presumably decreases the levels of Notch leading to less maintenance of the stem cell pool as such, allowing the stem cells to proliferate more efficiently into TA cells.

Compared to WT mice, the heterozygote mice yielded an intermediate amount of PHH3 at the protein level (Fig. 7A) once again emphasizing the dose-dependent impact of keratins. How the presence and absence of K8 affected the amount of PHH3 was also studied by immunofluorescence staining which confirmed the increased amount of PHH3 in the proliferative zone of the crypts of K8-null mice (Fig. 7C). Absence of K8 in mice causes colonic epithelial hyperproliferation (Habtezion et al. 2011) which seems to be a result of increased amount of TA cells and a widened proliferative zone (Fig. 7) most likely via reduced Notch signaling activity.

2.9 Conclusions of the interaction between Notch and keratins

The interaction between Notch and keratins is the most prominent discovery of project (II). New Notch-interacting proteins are still being discovered, being exemplified among others by the recently discovered chromatin-modeling protein Hat-trick (Singh et al. 2019). We presented evidence that keratins affect colonic epithelial cell differentiation by regulating different aspects of Notch. Keratins increase NICD levels (Fig. 2C, E, F, SI Fig. 3A-B, Fig. 4Eg), enhance Notch target gene Hey1/Hey2 expression (Fig. 2G), but do not affect NICD degradation speed (Fig. 2H), NICD turnover (SI Fig. 3A, C) or FLN turnover (SI Fig. 3D).

However, it is still unclear how keratins mechanistically regulate Notch. A potential possibility for how keratins mechanistically affect Notch signaling include that keratins enhance the transport of FLN to the plasma membrane. This could be studied by observing the cellular localization of FLN in the presence of different amounts of keratins, e.g. knockout and overexpression.

It could perhaps be speculated that keratins affect FLN stability. However, presence/absence of keratins did not seem to affect FLN turnover under many conditions of inhibiting protein translation by cycloheximide (SI Fig. 3D). For further elucidation on the potential effect of keratins on FLN stability, the degradation rate of FLN under conditions of exposing cells to the proteasome inhibitor MG132 in the presence of different amounts of keratins, could be studied. The degradation rate of NICD under the influence of MG132 was not affected by keratins (Fig. 2H).

Keratins have been demonstrated to target proteins at the cell membrane (Toivola et al. 2004; Vijayaraj et al. 2009). Therefore, a thinkable event is that keratins facilitate Notch cleavage at S3 cleavage site. If keratins enhance cleavage at S3 then increased amounts of keratins e.g. by overexpression should result in more free cytoplasmic NICD in relation to ΔEN. In contrast, reduced amounts of keratins e.g. by CRISPR/Cas9 or *in vivo* knockout should lead to more ΔEN in relation to free cytoplasmic NICD. This could be studied for instance with overexpression of tagged ΔEN. γ-secretase inhibitors preventing S3 cleavage could also be utilized to provide more settings for studying the effect of keratins on S3 cleavage.

Keratins prolonging NICD activity by retaining a pool of NICD in the cytoplasm and preventing nuclear degradation, or keratins aiding in the translocation of NICD to the nucleus are potential mechanisms in how keratins affect Notch signaling. The impact of keratins on Notch cellular localization could be studied, for instance as in Fig. 3 in project (I), with immunofluorescence confocal microscopy. Cells could be analyzed based on if Notch localization is strongly nuclear, equally nuclear/cytoplasmic or mostly cytoplasmic. Alternative methods include for instance live imaging of GFP-tagged constructs, and fluorescence recovery after photobleaching (FRAP), a method for determining the kinetics of diffusion in living cells normally using fluorescence microscopy. Additionally, cytoplasmic and nuclear fractions of cells could be extracted for analysis of the amount of Notch in these fractions in the presence/absence of different amounts of keratins.

Previously it was believed that keratins only exist in the cytoplasm, but relatively recently K17 was identified inside tumor epithelial cell nuclei with a direct after-effect on cell proliferation and gene expression (Hobbs et al. 2016). In the experiments where we studied the effect of keratins on the Notch signaling pathway by using overexpression of keratins (Fig. 1, 2), we overexpressed both K8 and K18 since these two proteins form heterodimers with each other and cannot thus function without the presence of the other. When studying the effect of K8/K18 on the Notch signaling pathway and consequently on proliferation and differentiation, by the absence of these two keratin isoforms, it was sufficient to cause the absence of specifically K8, either by K8 KO in mouse models (Fig. 3, 6, 7) or by CRISPR/Cas9 in cultured cells (Fig. 4, 5), since K18 is degraded without the possibility to pair with K8.

As stated already earlier, several keratin isoforms have been demonstrated to rapidly increase in amounts when subjected to cellular stress. These isoforms include at least K6a, K6b, K16 and K17 whose expression is enhanced by oxidative and UV stress, wounds and inflammatory cytokines (Haines and Lane 2012). Cellular stress in many cases also allows keratin filament reorganization. Both the increased amount and reorganization of keratins allow the cell to more efficiently battle the cellular stress in question (Omary 2017). In addition, during some stress conditions the expression of SEKs which include K8 and K18, is enhanced up to a 3-fold (Omary et al. 2009). In this thesis, I have stated that both SUMO proteins and keratin proteins not only directly interact with Notch, but the behavior of these two Notch-interacting proteins is impacted by a common nominator which is cellular stress in different forms. It could be contemplated whether cellular stress affects the interaction between keratins and Notch. This matter was also addressed in results & discussion section "2.6 Effect of K8 S74 phosphorylation on Notch signaling".

In the light of the role of cellular stress in the upregulation of keratin abundance during many circumstances (Omary 2017), and since we have shown that cell stress in the form of heat shock itself increases Notch target gene Hes1, Hey1 and Hey2 expression (SI Fig. 2B, study I), cellular stress may indirectly increase the amount of keratins being able to bind to Notch. This would in turn have an impact on Notch activity ultimately altering Notch target gene expression within the pathway, and consequently extrinsically alter cell fate of intestinal epithelial cells. Whether cellular stress has the capability to directly enhance the binding of keratins to Notch in a similar manner as heat shock enhances SUMO-Notch interaction, is more speculative.

The cytoskeletal protein network can interact with and therefore regulate different receptors through a variety of mechanisms. We found that keratins directly bind Notch. We used tissue samples and different cell lines with different amounts of keratins to study the effect of keratins on Notch and its target genes which regulate colonic proliferation and differentiation events. By carrying out both *in vitro* and *in vivo* experiments, our data showed that K8/K18 directly interacts with Notch. The *in vitro* and *in vivo* interactions were further supported by our observations that K8 and Notch were co-localized in the epithelial cells of the colon, enhancing Notch levels and target genes. Absence of K8/K18 on the other hand resulted in altered colonic epithelial cell differentiation towards a secretory cell fate, and to a widened proliferative zone and an increased amount of TA cells in the colon. The K8 heterozygous mice displaying an intermediate amount of keratins without developing inflammation, compared to WT and KO K8, also displayed an intermediate phenotype in terms of altered Notch signaling activity. The data presented in project (II) display that K8/K18 of the cytoskeletal network plays an integral role in regulating the proliferation and differentiation of colonic epithelial cells via the Notch signaling pathway. Hopefully this project will aid in understanding early steps and development of diseases such as IBD and CRC.

CONCLUDING REMARKS

The Notch signaling mechanism creates cellular diversity during development and promotes tissue integrity and homeostasis. In developmental biology, it is a challenge to understand how complex organs, tissues and entire organisms develop with such conscientiousness. It is also a challenge to gain detailed mechanistic insight into how developmental guides are regulated, their activity fine-tuned and how disturbances in these processes contribute to the development of human diseases. Signaling via the transmembrane receptor Notch is an important regulator of development and disease and a growing field within molecular cell biology.

The general knowledge of how signaling proteins are being modified by PTMs is constantly increasing. Among these modifications, protein sumoylation constitutes a mechanism for dynamic regulation of protein activity allowing the interest towards SUMO-regulated functions to increase. Protein-protein interactions constitute another important regulator of signaling activities and cell fate.

In this thesis we have unraveled a new molecular mechanism of Notch regulation linking stress-induced sumoylation to a key regulator of cell fate during development and progression of a variety of disease conditions. The presented work suggests a new layer of molecular control governing Notch signaling and demonstrates that sumoylation might be an integral mechanism regulating the Notch signaling cascade especially under conditions of cellular stress. Notch signaling is notoriously context-dependent and our new information on how Notch is regulated by sumoylation in cell stress significantly enhances the understanding of how Notch is influenced by the cellular state.

Notch is known for steering stem cells into different cell fates, and plays an important role in regulating homeostasis of the colon, where cytoplasmic keratins protect epithelial cell and tissue integrity. We show that K8/K18 regulate stem cell differentiation through the interaction with Notch and the regulation of Notch target genes. Keratins interact with Notch in the cytoplasm whereas the SUMO-Notch interaction takes place specifically in the nucleus giving rise to a potential cytoplasmic vs nuclear regulation of Notch target gene expression and consequently of Notch-mediated outcomes. Keratins are regulated by cell stress potentially enforcing cytoplasmic keratin-Notch interaction and Notch activity during stress. On the other hand, the nuclear SUMO-Notch interaction increases causing repressed Notch target gene expression. This might provide different fine-tuning of cell stress-mediated regulatory outcomes via the Notch signaling pathway that balance each other through different mechanisms.

We have only begun to understand the Notch-SUMO and Notch-keratin interplay. Dysregulations of these interactions are expected to cause intracellular malfunctions that may eventually lead to tissue malformation and disease. All three proteins have been associated with cancers although inconsistencies in the expression levels of Notch and the lack of mechanistic insight into Notch regulation create uncertainties about the function of Notch signaling in different tumorigenesis settings. The discoveries in this thesis contribute to the understanding of how Notch activity is regulated and how this impacts on cell fate in the CNS and the intestine. As both keratins and SUMO are stress sensors, our data also provide new knowledge on the context-dependency of Notch signaling and on the mechanisms that may tune Notch activity in response to different cellular states.

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