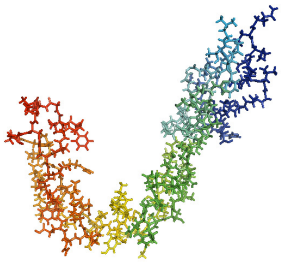
PROGRAM



The 4th International Conference
'Notch Targeting in Cancer'
Santa Marina Hotel, Mykonos, Greece
25 - 27 June 2014

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Wednesday 25th June 2014

4.00 - 4.30 pm Registration

4.30 - 4.35 pm Welcome: Agamemnon Epenetos

SESSION 1 Chairman: Christina Kousparou

4.30 - 5.00 pm Keith Brennan

Highlights of the 3rd Meeting, Notch Targeting in Cancer, June, 2013

5.00 - 5.30 pm Freddy Radtke , EPFL ,CH-1015 Lausanne, Switzerland

Molecular mechanisms controlling stem cell maintenance, lineage commitment and differentiation

5.30 - 6.00 pm Jon Aster , Department of Pathology, Brigham and Women's Hospital,77 Avenue Louis Pasteur, Boston, MA 02115, USA

Notch Signaling in Cancer: Predictors of Response and Resistance to Notch Pathway Inhibitors

Multiple human cancers are associated with gain-of-function mutations in various Notch receptors, observations that have stimulated interest in targeting this pathway therapeutically. Initial attempts to do so with gamma-secretase inhibitors (GSIs) were plagued by on-target toxicity, but subsequent trials have shown that GSIs are well tolerated when given on a staggered dosing schedule. The principle challenge now is to prove that GSIs and other Notch inhibitors can produce significant anti-tumor activity in patients with Notch-related cancers. We have recently focused on identification of biomarkers that predict tumor response in diverse mouse models of Notch-related cancers such as leukemia, breast cancer, and adenoid cystic carcinoma, and have observed that the most reliable marker is simply the level of activated Notch in tumor cells. We hypothesize that this reflects the remarkable capacity of Notch transcription complexes to influence chromatin marks across broad regulatory switches termed super-enhancers, which have recently been implicated in regulating the expression of

lineage-specific differentiation genes as well as oncogenes. This talk will highlight specific chromatin states that correlate with responsiveness and resistance to Notch pathway inhibitors, and will also discuss studies focused on understanding the basis for "super-responders", tumors in patients on clinical trials that have proven to be unusually sensitive to Notch pathway inhibitors.

8.00 - 10.00 pm
Welcome Reception Cocktail

Thursday 26th June 2014

SESSION 2 Chairman: Jon Aster

9.00 -9.30 am **Aleksandra Filipovic, He Zhu, Ingrid Espinoza, , Andrew Green and Lucio Miele, University of Mississippi Cancer Institute, Jackson, MS; Imperial College, London, UK; Cardinal Bernardin Cancer Center, Loyola University Medical Center, Maywood, IL; University of Florida, Gainesville, FL; Baylor University Medical College, Houston, TX; Stanley S. Scott Cancer Center, Louisiana State University Health Center and Louisiana Cancer Research Consortium, New Orleans, LA**

Correlation of Notch1 membrane and nuclear expression with clinical characteristics, signaling biomarkers and survival in breast cancer patients

Background: Notch1 signalling plays an important oncogenic role in breast cancer and represents a therapeutic target amenable to targeting with small-molecule gamma secretase inhibitors and specific anti-Notch1 monoclonal antibodies. Canonical Notch1 signalling is mediated by nuclear Notch1 cleaved fragment. Some evidence suggests non-nuclear, non-canonical signalling from cytoplasmic or membrane Notch1. Current study aims to investigate the clinical relevance of Notch1 expression in invasive breast cancer.
Methods: A well-characterised Nottingham Tenovus cohort of invasive breast cancers (n = 1078), with long-term follow up data, was analysed using immunohistochemistry for Notch1 (Santa Cruz, C-20). X-tile software was used to generate cut-off values for further correlation of the Notch1: nuclear (≥ 20% positive cells) and membrane/juxtamembrane staining (≥ 50% cells).

Results: High Notch1 membrane staining was correlated with high grade (p = 0.001), size > 2cm (p = 0.001), ductal histotype, advanced disease stage (p = 0.044), triple negative phenotype (p = 0.001), HER2 positive status (p = 0.02), HER3 positivity (p = 0.019), basal cytokeratin CK5 expression (p = 0.001) and PI3K activity (p = 0.008). High membrane Notch1 predicted worse breast cancer specific overall survival at 25 years of follow up (p = 0.017). Conversely, nuclear Notch1 was predominantly present in lobular carcinomas and correlated inversely with the triple negative subtype (p = 0.019) as well as PI3K (p = 0.014), thereby its higher expression was predictive of favourable survival outcome (p = 0.008). **Conclusions**: Different subtypes of breast cancer may rely on different Notch1 signalling pathways, including nuclear and non-nuclear signalling. For the first time, we emphasize the clinical relevance of Notch membrane expression in highly aggressive disease. Therefore, our finding bear significant translational relevance as we propose that Notch1 membrane expression assessment may be used for stratification of patient who may derive particular benefit from anti-Notch1 monoclonal antibody therapy or other Notch inhibitors in the clinic.

9.30 - 10.00 am Shuheng Lin[1], Ana Negulescu[1], Sirisha Bulusu[1], Benjamin Gibert[1], Benjamin Ducarouge[1], Jean-Guy Delcros[1], Nicolas Gadot[2], Olivier Meurette[1,$] and Patrick Mehlen[1,$]

[1]Apoptosis, Cancer and Development Laboratory- Equipe labellisée 'La Ligue', LabEx DEVweCAN, Centre de Recherche en Cancérologie de Lyon, INSERM U1052-CNRS UMR5286, Université de Lyon, Centre Léon Bérard, 69008 Lyon, France. [2]ANIPATH, Centre de recherche en Cancérologie de Lyon, INSERM U1052-CNRS UMR5286, Université de Lyon, Hospices Civils de Lyon, Hopital Edouard Herriot, Anatomie Pathologique, 69437 Lyon, France. [$]Co-senior and co-corresponding authors: P Mehlen; email: patrick.mehlen@lyon.unicancer.fr and O Meurette; email: olivier.meurette@lyon.unicancer.fr

NOTCH3 limits tumour angiogenesis through a non-canonical pro-apoptotic pathway

Notch signaling is a highly conserved pathway involved in development and cancer. In mammals four Notch receptors have been described and five canonical ligands Jagged-1 and 2 and Dll-1, 3 and 4 exist. Specific role of these different receptors are emerging but are still poorly understood. Notch3 has been shown to be essentially expressed in the vascuature and mutations of Notch3 are

responsible for Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL). More recently, Notch3 has been shown to be expressed in epithelial cells and to be involved in cancers where depending on the context, it can behave as an oncogene or as a tumour supressor. We decided to study Notch3 in tumour angiogenesis and we uncovered an unexpected role of Notch3 as a dependence receptor in regulating tumour angiogenesis. Using Notch3 knock-out mice and Lewis lung cancer (LLC1) syngenic graft, we showed that Notch3 was expressed in the tumour vasculature and we observed an increase in tumour vascularization when Notch3 was absent from the stroma of tumours. Moreover treatment with DAPT (a known inhibitor of Notch signaling that mimic absence of ligands) induced a decrease of tumour vasculature when Notch3 was expressed by the vasculature but not in the Notch3 knock-out mice. Similarly, *in vitro*, treatment of endothelial cells with DAPT induced cell death which was inhibited by knocking-down Notch3 but not Notch1 or Notch2. Furthermore, expression of Jagged-1 by cancer cells co-cultured with endothelial cells decreased apoptosis in endothelial cells and reduced Notch3-induced cell death. In human tumours, we showed that Jagged-1 was over-expressed in lung cancers and correlated with the endothelial marker CD31. We therefore hypothesise that Notch3 expressed in the vasculature limits tumour angiogenesis by inducing cell death which can be inhibited by Jagged-1 expressed by the epithelial compartment.

10.00 - 10.30 am Cristiana Caliceti[1], Micaela Pannella[1], Giorgio Aquila[1], Marco Bruno Morelli[1], Antonio Pannuti[2], Lucio Miele[2], Paola Rizzo[1,2] and Roberto Ferrari[1,2].

1 Department of Medical Sciences (Chair of Cardiology) and Laboratory for Technologies of Advanced Therapies (LTTA Center), University of Ferrara, Ferrara, Italy

2 Maria Cecilia Hospital, GVM Care&Research, E.S: Health Science Foundation, Cotignola, Italy.

3 Louisiana State University Health Sciences Center New Orleans, Louisiana. USA

Corresponding author: Dr. Cristiana Caliceti email: cristiana.caliceti@gmail.com

17β-Estradiol Enhances Notch1 Activation and modulates angiogenesis through Estrogen Receptor β in Human Vein and Artery Endothelial Cells

Background: Many studies have shown that estrogens play a protective role in the cardiovascular system. The mechanisms of action are still poorly understood,

although a role for estrogens in stimulation of angiogenesis has been suggested. Notch1 modulates angiogenesis also by inducing endothelial nitric oxide production. TNFα affects angiogenesis by inhibiting Notch. We previously demonstrated that 1) 17β-estradiol (E2) specifically enhances the VEGFA-Dll4-mediated activation of Notch signaling in human umbilical vein endothelial cells (HUVECs) and 2) E2 counteracts the increase in endothelial cells sprouting caused by the γ-secretase inhibitor DAPT, suggesting that E2 modulates angiogenesis in conditions of low levels of active Notch1. Estrogen receptors (ER) antagonist ICI 182.780 partially inhibits E2-induced Notch1 activation suggesting that the action of E2 is mediated by one or both ERs (α and β). Our report is in contrast with findings of Notch1 inhibition by E2 treatment in ERα positive breast cancer cells; since endothelial cells differently from breast cancer cells express high levels of ERβ, we hypothesized that these opposite results could be due to the different activity of the two forms of ERs on Notch1. The aim of this works was 1) to investigate a possible differential modulation of Notch activity by specific ER agonists; 2) to establish whether E2 could counteract Notch inhibition induced by TNFα and to investigate whether this regulation is specific to HUVECs or it is present also in artery endothelial cells.

Results: Treatment with ERβ specific agonist (DPN) but not with ERα specific agonist (PPT) induced activation of Notch1 in HUVECs, HCAECs (human coronary artery endothelial cells) and HAECs (human aortic endothelial cells). Human endothelial cells tube formation assay showed that *i*) DPN counteracted the increase in endothelial cells sprouting caused by DAPT; *ii*) ICI 182.780 partially antagonized the effects of E2 on DAPT- mediated increase in sprouting of endothelial cells. Interestingly, E2 or DPN treatment antagonized TNFα-induced decreased of cleaved Notch1 and eNOS.

Conclusion: Our data confirm and expand our previous observations showing activation of Notch1 by E2 and suggest that ERβ might be implicated in E2-mediated activation of Notch1 processing in artery and vein endothelial cells as well as Notch regulation of angiogenesis. Of interest DPN treatment partially counteracted Notch1 and eNOS inhibition induced by TNFα treatment. More studies are needed to establish if DPN restores eNOS levels by activating Notch.

SESSION 3 Chairman: Freddy Radtke

11.30 - 12.00 noon Ulrike Harjes, Esther Bridges, Alan McIntyre, Barbara A.Fielding, Adrian L. Harris , University Department of Oncology Weatherall Institute of Molecular Medicine, John Radcliffe Hospital Oxford OX3 9DS

DLL4-NOTCH and FOXO1 regulate endothelial Fatty Acid Binding Protein 4 downstream of VEGFA

Fatty acid binding protein 4 (FABP4) is an adipogenic protein and implicated in atherosclerosis, insulin resistance, and cancer. In endothelial cells, FABP4 is induced by VEGFA and inhibition of FABP4 blocks most of the VEGFA effects. We investigated the DLL4-NOTCH-dependent regulation of FABP4 in human umbilical vein endothelial cells (HUVECs) by gene/ protein expression and interaction analyses following inhibitor treatment and RNA interference.
We found that FABP4 is directly induced by NOTCH. Stimulation of NOTCH signalling with human recombinant DLL4 led to FABP4 induction, independently of VEGFA. FABP4 induction by VEGFA was reduced by blockade of DLL4 binding to NOTCH, or inhibition of NOTCH signal transduction. Chromatin immunoprecipitation of NOTCH intracellular domain showed increased binding to two specific regions in the *FABP4* promoter. The induction of *FABP4* gene expression was dependent on the transcription factor FOXO1, which was essential for basal expression of FABP4, and FABP4 upregulation following stimulation of the VEGFA and/or the NOTCH pathway.
Thus we show that the DLL4-NOTCH pathway mediates endothelial FABP4 expression. This indicates that induction of the angiogenesis-restricting DLL4-NOTCH can have pro-angiogenic effects via this pathway. It also provides a link between DLL4-NOTCH and FOXO1-mediated regulation of endothelial gene transcription, and shows that DLL4-NOTCH is a nodal point in the integration of pro-angiogenic and metabolic signalling in endothelial cells. This may be crucial for angiogenesis in the tumour environment.

12.00 - 12.30 pm Jing Shan Lim, Angela Torres, Dedeepya Vaka and Julien Sage, Cancer Biology Program, Departments of Pediatrics and Genetics, Stanford University, Stanford, CA, USA

Temporal-specific effects of Notch activation in small cell lung cancer

Small cell lung cancer (SCLC), a very aggressive neuroendocrine (NE) tumor of the lung, constitutes approximately 15% of all lung cancers diagnosed and has a 5-year survival rate of only 5%. Resistance following initial response to chemotherapy and radiation therapy accounts for the high rate of recurrence and the overall poor prognosis of SCLC. Studies of the molecular and cellular mechanisms underlying SCLC development would help identify targeted therapies against SCLC. Depending on the cellular context, the Notch pathway can act in either an oncogenic or a tumor suppressive manner. Notch pathway components are minimally expressed in NE tumors and emerging evidence suggests that hyperactivation of the pathway inhibits the growth of human SCLC cell lines. Based on these findings, activation of Notch has been proposed as a therapeutic strategy for SCLC.

To study the role of the Notch pathway in SCLC *in vivo*, we bred mice carrying conditional alleles for active forms of either Notch1 (N1ICD) or Notch2 (N2ICD) to a mouse model for SCLC−*Rb/p53/p130* conditional triple knockout (TKO) mice that develop SCLC tumors after intratracheal administration of the Cre recombinase.

At an early time point (3 months post-Cre), TKO;NICD mice develop significantly fewer and smaller lesions compared to TKO mice, indicating that activation of Notch strongly inhibits the initiation of SCLC. Accordingly, TKO;NICD mice survive longer post-Cre as compared to TKO controls. Interestingly, however, some tumors grow in TKO;NICD mice. These tumors have gross histopathological features of SCLC but do not stain positive for neuroendocrine markers. In addition, TKO;NICD tumors are significantly more proliferative compared to TKO tumors. Similarly, while the expansion of human SCLC cells is initially inhibited by active Notch, rapidly growing cells emerge from these experiments.

These observations suggest that activation of the Notch pathway may be tumor suppressive at the early stages of SCLC or acutely in established tumors, but possibly pro-oncogenic during SCLC progression. Current studies are focused on dissecting the mechanism by which this temporal difference in the mode of action of Notch occurs, and the response of the tumors to chemotherapy.

12.30 - 1.00 pm Marianna Prokopi[1], Christina A. Kousparou[1] & Agamemnon A. Epenetos[1, 2]
1. Trojantec Ltd, The Bank of Cyprus Oncology Centre, 32 Acropoleos Avenue, 2006, Nicosia, Cyprus
2. Imperial College London, London, UK

The secret role of microRNAs in cancer stem cell development: The NOTCH-pathway

MicroRNAs (miRNAs) have been implicated in the development of some if not all cancer types and have been identified as attractive targets for prognosis, diagnosis and therapy. MiRNAs are a class of small non-coding RNAs (20-22 nucleotides in length) which bind imperfectly to the 3'-untranslated region of target mRNA regulating gene expression. Aberrantly expressed miRNAs in cancer can function either as oncogenes or tumor suppressor genes and are therefore referred to as 'oncomiRs'. OncomiRs play a major role in cancer pathogenesis, tumor formation, metastasis and therapy resistance.

- Amplification of miRNAs (oncogenes) during cancer development correlates with the silencing of tumor suppressor genes, and down-regulation of miRNAs (tumor suppressor genes) is commonly observed in tumor cells and cancer stem cells (CSCs), thus miRNA down-regulation is inversely correlated with cancer progress.
- Growing evidence indicates that oncomiRs are involved in the metastatic process regulating this by either suppressing or promoting metastatic-relating genes leading in the reduction or activation of cancer cell migration and invasion.
- Circulating oncomiRs (vesicle-encapsulated or non-encapsulated) have significant effects on tumorigenesis: membrane-particles, apoptotic bodies and exosomes have been described as providers of a cell-to-cell communication system transporting oncomiRs from tumors to neighbouring cells and distant metastatic sites.
- Thus miRNAs control cancer progress conventionally by regulating signaling pathways and factors.
- However, recent developments indicate a non-conventional mechanism of cancer regulation by CSC reprogramming via a regulatory network consisting of miRNAs and Wnt/β-catenin, Notch, and Hedgehog signaling pathways

In this review, we focus on the role of miRNAs in the Notch pathway and how they regulate cancer, CSCs self-renewal, differentiation and tumorigenesis by direct/indirect targeting of the Notch pathway components. A delivery mechanism of therapeutic miRNAs is proposed.

1.00 - 2.30 pm **Lunch**

2.30 - 3.30 pm **Open Air workshops:**
'Notch - Friend or Foe' Adrian Harris/ Olivier Meurette

SESSION 4 Chairman: Adrian Harris

3.45 - 4.15 pm **Bruno Simões, Ciara O'Brien, Rachel Eyre, Ling Yu, Andreia Silva, Denis Alferez, Kath Spence, Ahmet Acar, Julia Gee, Keith Brennan, Andy Sims, Elisabetta Marangoni, Goran Landberg, Sacha Howell and Robert Clarke**
Breast Biology Group, Institute of Cancer Sciences, University of Manchester

Notch4 activation regulates endocrine therapy-induced enrichment of breast cancer stem cells

The majority of breast cancers express the estrogen receptor (ER), and respond to the anti-proliferative effects of endocrine therapy. However, hormone-responsive breast cancers frequently develop resistance to hormonal therapies. The potential involvement of breast cancer stem cells (CSCs) in endocrine resistance makes it imperative to understand the cellular signalling pathways that could be targeted to eradicate breast CSCs and, therefore, provide long-term disease-free survival. One strong candidate for is the Notch pathway, which is known to be activated in breast CSCs.
We observe that the anti-estrogens tamoxifen and fulvestrant increase the percentage of breast CSCs *in vivo* in ER-positive patient-derived xenograft (PDX) tumours by determining the mammosphere formation efficiency, ALDH enzymatic activity and tumour initiation capacity after treatment. We found that both tamoxifen and fulvestrant increase Notch transcriptional activity via Notch4 and up-regulate Notch target genes, which correlate with ALDH activity. We find that both ALDH1 protein staining and a Notch4 gene signature predict tamoxifen resistance in patients suggesting this is of clinical importance. Finally, we used a gamma-secretase inhibitor (RO4929097), which inhibits Notch activity, to abrogate the CSC increase induced by anti-estrogens. Our results thus far suggest that combining standard endocrine therapies with drugs targeting Notch signalling will be effective in overcoming endocrine therapy resistance of ER-positive breast cancer.

4.15 - 4.45 pm Kinnari Pandya, Debra Wyatt, Brian Gallagher, Deep Shah, Andrew Baker, Steven James, Andrei Zlobin, Lucio Miele, Mitch Denning, and Clodia Osipo, The Stritch School of Medicine, Cardinal Bernardin Cancer of Loyola University Chicago.

Novel Role for HER2-PKCα Axis on Jagged-1-mediated Notch Activation in HER2+ Breast Cancer: Implications for Therapeutic Interventions

We have demonstrated that Notch1 is required for trastuzumab resistance in HER2 positive breast cancer. Our data indicates that HER2 suppresses Notch1 in breast cancer and therapeutic intervention targeting HER2 might have an unintended consequence which is aberrant up regulation of Notch1, a potent breast oncogene. However, the mechanism of action by which HER2 restricts Notch1 activation is unknown. In this current study, we investigated the role of Jagged-1 on trans-activation of Notch signaling to address this question. We performed co-culture studies using fibroblasts expressing no Notch ligands or over-expressing human Jagged-1 or Deltalike1 and HER2 positive breast cancer cells. We performed flow cytometry to isolate breast cancer cells after co-culture and extracted RNA to measure expression of Notch gene targets as a measure of Notch activity. The results showed that trastuzumab, Lapatinib, or HER2 knockdown increased overall Notch activation. Similarly, co-culture with Jagged-1-expressing fibroblasts increased overall Notch activation. Jagged-1 knocked down abrogated trastuzumab-induced Notch activation in the breast cancer cells and enhanced trastuzumab's therapeutic efficacy in vitro and in vivo. These results suggest that HER2 might restrict Notch activation by preventing Jagged1-mediated trans-activation of Notch. Confocal immunofluorescence showed that Jagged1 is localized with Notch1 when HER2 is hyperactive but is trafficked to the cell surface in response to trastuzumab. K44ADynamin abrogated Jagged1 expression on the cell surface as measured by IF and surface biotinylation studies. Furthermore, K44ADynamin expression abrogated trastuzumab-induced Notch1 activation. The critical role of endocytosis on Notch activation implied that HER2 could be regulating the competency of Jagged-1. We therefore investigated whether HER2 is attenuating Mib-1's ability to ubiquitylate Jagged1 and thus restrict Notch activation. For the first time, we showed that targeting HER2 with trastuzumab or lapatinib increases the interaction of MIb-1 with Jagged-1 and enhances ubiquitylation of Jagged-1 to promote trans- activation of Notch. Importantly, we identified that PKCα downstream of HER2 is both necessary and

sufficient to restrict the interaction of Mib-1 with Jagged-1, ubiquitylation of Jagged-1, and Notch activation. Kaplan-Meier analysis of 193 HER2+ patient data demonstrated that low PKCα, high Jagged-1, high HES1, and high HEY1 expression significantly correlates with poor recurrence free survival. These data taken together suggest that PKCα could predict for Notch activation state and importantly trastuzumab resistance in HER2+ breast cancer.

4.45 - 5.15 pm Keith Brennan, Lorna Wilkinson, Stephanie Jobling, Michael Leverentz, Sally Wood, Olivier Meurette, Spyros Stylianou
Faculty of Life Sciences,University of Manchester, Manchester, Oxford Road, Manchester,M13 9PT

Activating RBPj-dependent Notch signalling in the murine mammary gland leads to spontaneous endocrine resistant ER+ve tumours

Aberrant Notch signalling has been shown to play a causal role in human breast cancer. Increased signalling is seen in a wide variety of breast cancers, including ductal carcinoma in situ where it is indicative of lesions that are likely to recur. Also sustained Notch signalling has been shown to increase proliferation, prevent apoptosis, promote stem cell self-renewal and drive the adoption of a mesenchymal morphology in breast cancer cells. However it is not clear how increased activity of the Notch receptors is linked to changes in these cellular properties. Typically the Notch receptors are thought to signal via the nuclear protein RBPj, but there is clear evidence that an active form of Notch4 can transform mammary epithelial cells in the absence of RBPj in the mouse. To address the possible role of RBPj-dependent Notch signalling in breast cancer, we have generated a transgenic mouse strain expressing an activated form of RBPj under the control of the mouse mammary tumour virus promoter. These mice display a failure in ductal outgrowth during puberty with increased ductal branching and enlarged terminal end buds, as there is a change in the direction of cell division within the end buds. The mice progress normally through lactation but display a delayed involution. They also develop high grade papillary adenocarcinoma with a latency of 10-12 months that are associated with ductal carcinoma in situ lesions. By isolating primary mammary epithelial cells and tumour cells from these mice, we have been able to demonstrate that RBPj-dependent Notch signalling induces a significant resistance to a range of apoptotic stimuli that is dependent on Akt signalling. We also found that the

proliferation of the tumour cells was resistant to anti-oestrogens unlike contralateral cells. Together these data indicate that RBPj-dependent Notch signalling can cause tumour formation in the mammary gland, leading to an acquired apoptosis and anti-oestrogen resistance.

8.30 pm until late
Conference Dinner

Friday 27th June 2014

SESSION 5 Chairman: Aleksandra Filipovic

9.30 - 10.00 am Marika Sjöqvist[1,2], Daniel Antfolk[1,2] & Cecilia Sahlgren[1,2,3]
[1] Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, 20520 Turku, Finland
[2]Department of Biosciences, Åbo Akademi University, 20520 Turku, Finland
[3] Department of Biomedical Engineering, Technical University of Eindhoven, 2612 Eindhoven, The Netherlands

Atypical PKCζ regulates Notch receptor routing and activity in a Notch signaling-dependent manner

The atypical PKCs are components of the PAR3/PAR6/aPKC cell polarity complex. They play critical roles in asymmetric cell division, migration and also link cell polarity cues to differentiation. Aberrant expression of protein kinase C family isozymes is associated with different human cancers. Expression of aPKC is increased in non-small cell lung carcinomas and colon carcinomas, promoting cancer development and chemotherapy resistance. aPKC regulates e.g. epithelial-to-mesenchymal transition, a distinct feature of metastasis.
Here we present PKCζ as a regulator of Notch intracellular routing and signaling output. PKCζ modulates the function and activity of Notch by site specific phosphorylation of the receptor. Membrane tethered Notch receptors are subjected to phosphorylation and the outcome is dependent on the Notch activation state. Phosphorylation of activated Notch receptors results in translocation of Notch from late endosomes to the nucleus followed by increased production of Notch intracellular domain (NICD). Whereas in the non-activated

state, phosphorylation by PKCζ promotes internalization of Notch receptors from the membrane, followed by ubiquitylation and interaction with Hrs. Inhibition of PKCζ increases differentiation of the chick nervous system (CNS) *in vivo* and myogenic progenitors *in vitro* despite of Notch. This suggests that Notch is unable to keep the cells in an undifferentiated state in the absence of PKCζ. Our finding links together two central players in differentiation, and provides a new approach on Notch regulation that is also relevant in cancer.
PubMed: http://www.ncbi.nlm.nih.gov/pubmed/24662486

10.00 - 10-30 am Assaker G, Gerby B, Hoang T, Emery G
Institute for Research in Immunology and Cancer (IRIC), Université de Montréal, Montréal, Canada

Identification of novel regulators of the Notch ligands Delta-Like 1 and 4 as potential therapeutic targets for T-cell Acute Lymphoblastic Leukemia (T-ALL)

Introduction: Notch signalling pathway plays a key role in stem cell self-renewal, cell proliferation, and differentiation. Consequently, it has important developmental functions, and its aberrant activation leads to many diseases and cancers, including T-cell Acute Lymphoblastic Leukemia (T-ALL). Understanding how Notch signalling is regulated is thus central to understand cancer development and to identify new potential therapeutic targets. The Notch receptor responds to transmembrane ligands of the DSL family (Delta/Serrate/Lag-2) and many cancers involving Notch are ligand-dependent. For instance, activating mutations in NOTCH1 have been identified in over 55% of T-ALL, with 40% of these mutations resulting in ligand hypersensitivity or ligand-independent Notch activation. Strikingly, the molecular mechanisms that regulate ligand activation in the signal-sending compartment are yet to be fully characterized. Therefore the aim of this project is to identify regulators of the ligand Delta activity that subsequently modulate physiological Notch signaling, as well as its oncogenic activity in T-ALL.

Methods and Results: To unravel new Delta regulators, we performed a genome-wide shRNA screen using an in vitro co-culture assay, and targeting specifically the signal-sending cells. We used the OP9 mouse stromal cells stably expressing the ligand Delta-like 1 (DL1) as signal-sending cells, and HeLa cells stably transfected with a luciferase reporter of Notch as signal-receiving cells. This screen allowed us to identify 166 primary hits, which were further confirmed in OP9 cells expressing

another DSL ligand: DL4. Using functional secondary assays, we found that those hits are required for Notch-mediated events such as differentiation of hematopoietic progenitors into T-cells, and maintenance of pre-leukemic stem cells (pre-LSCs) isolated from a T-ALL mouse model. Moreover, we showed that some newly identified regulators are necessary for the progression of pre-leukemic T-cells into leukemic T-cells in vivo, following engraftment. This latter experiment demonstrates that pre-LSCs require ligand activation of Notch to induce leukemia, and validates our hits as potential molecular targets for the treatment of T-ALL.

Conclusion and Relevance: Altogether, our screen and the validation experiments allowed us to identify new classes of Notch ligand regulators. These could serve potentially to develop novel therapeutic strategies targeting the stromal microenvironment in Notch cancers, as exemplified by the T-ALL model.

10.30 - 11.30 am **Coffee Break and Poster Review**

SESSION 6 Chairman: **Robert Clarke**

11.30 -12.00 noon **Yijie Gao[1], Kenneth G. Geles[2], Joel Bard[2], Marc Damelin[2], Riyez Karim[1], Ping Wei[3], Lioudmila Tchistiakova[1] and Bin-Bing Zhou[2] Pfizer Worldwide Research and Development, [2]Global Biotherapeutic Technologies, Cambridge, MA, Oncology Research Unit, [1]Pearl River, NY 10968 and [3]La Jolla, CA 92121**

Discovery of a Notch1 inhibitory antibody with preferential activity against mutant receptor in T-ALL

The Notch1 receptor regulates cellular proliferation, differentiation, and survival through its intracellular domain, a transcriptional activator. Notch1 signaling plays essential roles in normal tissues processes such as cell fate specification, stem cell maintenance and angiogenesis. Compelling evidence has implicated Notch1 mutation and/or signaling dysregulation in both hematologic and solid tumors. We have developed a panel of potent and selective anti-Notch1 antibodies that inhibit ligand-dependent signaling by stabilizing the NRR domain in an auto-inhibited state thus preventing proteolytic cleavage and release of the Notch1 intracellular domain. T-cell leukemias often harbor mutations within the NRR of Notch1 and potentially escape targeting with an anti-Notch1-NRR

inhibitory antibody strategy. However, biochemical, structural and cell based analyses have indicated that our anti-Notch1-NRR antibodies are able to inhibit ligand-induced and constitutive signaling from wild-type as well as certain mutant Notch1 receptors. Moreover, one such antibody exhibited more potent inhibition activity against mutant Notch1 NRR in T-ALL with reduced activity against wild type Notch1. This differentiated inhibition profile was confirmed in *in vivo* efficacy studies. Co-crystallography and affinity analysis revealed potential insights into the mechanisms of this differentiated activity against wild type and mutant Notch1 receptors. This unique antibody activity profile may potentially provide a strategy to target mutant Notch1 in T cell leukemia with a better safety profile.

12.00 - 12.30 pm ***Zhu H[1], *Filipovich A[2], Pannuti A[1], Espinoza I[1], Osipo C[3], Osborne BA[4] ,Golde T[5], Fuqua S[5], *Albain K[3], Miele L[6]***
[1]University of Mississippi Cancer Institute, Jackson, MS; [2]*Imperial College, London, UK; *[3]Cardinal Bernardin Cancer Center, Loyola University Medical Center, Maywood, IL; [4]University of Florida, Gainesville, FL; [5]Baylor University Medical College, Houston, TX; [6]Stanley S. Scott Cancer Center, Louisiana State University Health Center and Louisiana Cancer Research Consortium, New Orleans, LA
***Contributed equally**

A COMPREHENSIVE STUDY OF NOTCH-RELATED BIOMARKERS IN THE NOTTINGHAM BREAST CANCER COHORT

BACKGROUND: Notch-1 and Notch-4 are mammary oncogenes, and are thought to play important but different roles in the pathogenesis and/or progression of endocrine-resistant ER+ breast cancer, Her2/Neu+ breast cancer and triple-negative breast cancer (TNBC). We have recently shown that PKCα modulates the expression of Notch-4 in endocrine-resistant breast cancer and that in a small cohort of TNBC, Notch-1 expression correlated with nuclear p65 (Rel-A NF-κB). Notch ligand Jagged1 and Notch1 have been implicated in the pathogenesis of TNBC. Notch-1 and its ligands are also involved in the modulation of cell fate decisions in multiple cell types in the immune system that may affect the tumor micro-environment. We analyzed a set of tissue microarrays (TMAs) from the well-characterised Nottingham Tenovus cohort of invasive breast cancers (n = 1078), with long-term follow up data. We used immunohistochemistry for Notch-

1, Notch-4, Jagged-1, PKCα and nuclear p65. X-tile software was used to generate cut-off values for further correlations. We correlated expression of these Notch-related biomarkers with other clinico-pathological parameters.

RESULTS: High Notch-1 membrane staining strongly correlated with high grade ($p = 0.001$), size > 2cm ($p = 0.001$), ductal histotype, advanced disease stage ($p = 0.044$), triple negative phenotype ($p = 0.001$), HER2 positive status ($p = 0.02$), HER3 positivity ($p = 0.019$), basal cytokeratin CK5 expression ($p = 0.001$) and PI3K activity ($p = 0.008$). High membrane Notch-1 predicted worse breast cancer specific overall survival at 25 years of follow up ($p = 0.017$). Conversely, nuclear Notch-1 was predominantly present in lobular carcinomas and correlated positively with androgen receptor (AR) expression and nuclear STAT3, while it correlated inversely with the triple negative subtype ($p = 0.019$) as well as PI3K ($p = 0.014$). Membrane Notch-1 was also correlated with nuclear p65. Additionally, membrane Jagged-1 was strongly correlated with poor survival. Interestingly, membrane Jagged-1 was also correlated with the presence of CD3+ and FoxP3+ T-cells in tumor stroma, suggesting a potential intra-tumoral immune modulatory role. Nuclear PKCα expression correlated strongly with nuclear Notch-4, in agreement with our published mRNA expression data. Nuclear Notch-4 also correlated with ER+ status ($p <0.005$) and STAT3 ($p = 0.001$).

CONCLUSIONS: Notch signaling may have different mechanistic roles in different subtypes of breast cancer. Membrane Jagged1 and membrane/cytoplasmic Notch-1 correlate with TNBC and Her2 status, aggressive clinical behavior and perhaps an immune-suppressive tumor micro-environment. Nuclear Notch-1 and Notch-4 are more clearly correlated with ER+ tumors. PKCα correlates with nuclear Notch-4. The correlation between non-nuclear Notch-1 and nuclear p65 suggests that non-nuclear, non-canonical Notch signaling pathways, already demonstrated in vitro and in T-cells, may play a role in the biology of some subtypes of breast cancer.

Funding NCI (LM, BAO, TG, AP).

12.30 - 1.00 pm Jan Kitajewski, PhD, Thaned Kangsamaksin PhD, Natalie Kofler PhD, Columbia University Medical Center, USA

Therapeutic targeting of ligand-specific Notch function

The Notch signaling pathway is a key regulator of both developmental and tumor

angiogenesis. Inhibition of Dll4-mediated Notch signaling in tumors results in hyper-sprouting of non-functional vasculature, paradoxically leading to poor tumor growth because the newly growing vessels are not functional. To explore the potential for targeting Notch in tumors we developed Notch inhibitors, the Notch1 decoys. Notch1 decoy variants have been developed that selectively inhibit DLL4 or Jagged1, identifying domains of Notch1 for ligand selectivity. These Notch1 decoy variants can disrupt tumor angiogenesis and inhibit tumor growth in several murine tumor models. A Notch1 decoy variant that inhibits Jagged1 reduces tumor vasculature and disrupts pericyte interaction with tumor endothelium, implicating Jagged in pericyte function. Endothelial cells and pericytes express Notch1 and Jagged1, whereas Notch3 expression is restricted to pericytes. Using genetic mutants, we found that Notch signaling is essential for pericyte function, and thus vessel maturation. Notch1 and Notch3 deficient mice display impaired pericyte/endothelial cell association in the developing retina. This phenotype was accompanied by retinal arteriovenous malformations, characterized by dilated vessels, vascular tangles and arteriovenous shunts. Thus, either pharmacological inhibition of Jagged-mediated Notch signaling or genetic deficiencies of Notch1 and Notch3 lead to pericyte dysfunction.

1.00 - 2.30 pm **Lunch**

2.30 - 3.30 pm **Open Air workshops:**
Notch Therapeutics Lucio Miele/Rob Clarke

SESSION 7 Chairman: Agamemnon Epenetos

3.30 - 4.00 pm Rajwinder Lehal[1], Viktoria Reinmüller[1,2], Viktoras Frismantas[3], Vincent Zoete[4], Olivier Michielin[4], Sina Reckel[1], Oliver Hantschel[1], Romain Hamelin[1], Gerardo Turcatti[1], Jieping Zhu[2], Jean-Pierre Bourquin[3] and Freddy Radtke[1]

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[3]University Children's Hospital Zurich, Division of Pediatric Oncology, August-Forelstrasse 1, 8008 Zürich, Switzerland

[4]Swiss Institute of Bioinformatics, Quartier Sorge - Batiment Genopode, 1015 Lausanne, Switzerland

Identification and pre-clinical validation of the novel Notch inhibitor I3

Notch signaling represents a type of cell-to-cell communication that plays a critical role in both embryonic development and adult tissue homeostasis. Over activation of the Notch cascade has been associated with cancer, including T-cell leukemia and breast cancer. Studies in mice and humans over the last two decades highlighted the critical need of targeting the Notch pathway in order to treat human malignancies. Current approaches to target Notch signaling are limited to small molecule g-secretase inhibitors (GSIs) and functional inhibitory antibodies against Notch receptors and ligands. Other therapeutic strategies including small inhibitory peptides are still in development.
The lack of effective therapeutic possibilities to directly counteract deregulated Notch signaling justifies the development of novel inhibitors. In order to identify Notch inhibitors, we have established a luciferase reporter assay to screen libraries of small molecules and peptides. This led to the identification of several Notch signaling inhibitors, of which we present here a small molecule, referred to as I3. The compound I3 has shown remarkable ability to block Notch signaling in human cancer cell lines as well as primary human T-ALL cells, thus abrogating their proliferative properties. In addition, compound I3 exhibits *in vivo* activity as demonstrated by its ability to affect Notch dependent developmental processes and by impeding tumour growth in xenograft models of human leukemia.

However, the mode of action is still unknown. For this purpose, we synthesized a comprehensive library of I3 derivatives in order to systematically investigate the structure-activity relationship. This enabled us not only to identify further Notch-inhibiting compounds, but also revealed at which position the molecule preferably tolerates an affinity tag, such as biotin. Currently we are using a biotinylated derivative of I3 to perform pull down experiments combined with mass spectrometry analysis to identify the target proteins of I3. Preliminary results support our hypothesis that I3 functions in the nucleus by interfering with proteins of the Notch transcription complex.

4.00 - 4.30 pm Krishna Polu, Jason Sagert, Jim West, Chihunt Wong, Annie Yang, Michelle Yang, Kenneth Wong, Elizabeth Menendez, Luc Desnoyers, Olga Vasiljeva, Jennifer Richardson, Henry Lowman, Jon Terrett,
CytomX Therapeutics, South San Francisco, CA

Targeting the Jagged Ligands for the Treatment of Cancer: A Probody Drug Conjugate Approach

The development of antibody drug conjugates (ADCs) holds significant promise for improving outcomes in patients with cancer. However, toxicity can limit the number of accessible targets for these highly potent and empowered antibody formats due to expression of such targets in healthy tissue. Probody formatted ADCs enable opening up the therapeutic window for high potential but previously inaccessible targets, such as the Jagged ligands in the Notch pathway. Probodies are fully recombinant biotherapeutics comprised of a monoclonal antibody whose binding to target antigen is blocked by a masking peptide that is joined to the antibody by a specific protease substrate-containing linker. Upon cleavage of the linker by tumor-specific proteases, the activated Probody therapeutic binds its target, resulting in tumor-localized activity. Jagged dependent Notch signaling and high expression of Jagged ligand on tumor cells has been described in a number of patient tumors including prostate cancer, squamous cell cancers, pancreatic cancer, and breast cancer. We previously described a novel anti-Jagged 1/2 antibody that can inhibit the Notch pathway and is efficacious in slowing tumor growth in a mouse in-vivo tumor pancreatic cancer model but results in systemic toxicity. A corresponding Probody therapeutic mitigates systemic toxicities associated with inhibition of Jagged-induced Notch signaling while maintaining anti-tumor efficacy in the same model.

Here we show that Notch ligands Jagged 1 and Jagged 2 have properties that could also enable an antibody-drug conjugate (ADC) approach because the ligands are both expressed on the cell surface and can internalize an anti-Jagged antibody. We have shown, using FACS, that Jagged 1 and Jagged 2 are expressed on many human cancer cell lines and, by fluorescent IHC staining, that the expression of Jagged 1 and Jagged 2 is maintained in the corresponding xenograft tumors. To further explore the potential of Jagged as an ADC target, we engineered a Probody Drug Conjugate (PDC) where the Probody component is conjugated to a microtubule inhibitor (MTI). This PDC is efficacious in pancreatic xenograft tumor model BxPC3. Importantly, in the BxPC3 model the PDC shows similar in vivo efficacy to the corresponding ADC without causing the systemic toxicity associated with ADC treatment. The full benefit of an anti-Jagged PDC may be optimized with alternative linker-toxin combinations.

Supportive of the potential clinical benefit of an anti-Jagged PDC, we have performed IHC on Tissue Microarrays of patient tumor biopsies using an anti-Jagged-1 mAb reagent and have observed significant membrane expression particularly in esophageal, HNSCC, and advanced prostate cancer. These data demonstrate that the Probody™ platform has the potential to enable the use of drug conjugates to target Jagged ligands in the Notch pathway in a number of different cancer types.

4.30 -5.00 pm **Wan-Ching Yen OncoMed Pharmaceuticals, Inc.**

Notch pathway inhibitors in cancer stem cells and angiogenesis

The Notch pathway is comprised of four receptors (NOTCH1-4) and five ligands (JAG1, JAG2, DLL1, DLL3, and DLL4), and is one of several key pathways linked to both stem cell biology and cancer. Although inhibition of Notch receptor cleavage enzymes by gamma-secretase inhibitors (GSIs) have been developed and progressed to the clinic, the therapeutic utility of these compounds is limited due to intestinal toxicity resulting from pan-Notch inhibition. We have developed monoclonal antibodies that selectively target either Notch the Ligand DLL4 or Notch2 and Notch3 receptors. Both antibodies demonstrate broad-spectrum anti-tumor activity against tumors of breast, colon, lung, ovarian and pancreas. In the tumor cells, targeting DLL4 or Notch2/3 signaling by either antibody inhibits cancer stem cell growth, promotes cell differentiation and delays tumor

recurrence following termination of chemotherapy. In the host stroma, anti-DLL4 antibody disrupts angiogenesis by inducing non-functional hyper-proliferative endothelial cells, whereas anti-Notch2/3 treatment modulates intratumoral pericyte localization and increases vessel perfusion while reducing tumor hypoxia. Collectively, these findings demonstrate that targeting Notch ligands or receptors affects tumor microenvironment, inhibits tumor growth and reduces cancer initiating frequency.

5.00 - 5.30 pm Yijie Gao[1], Kenneth G. Geles[2], Riyez Karim[1], Nicole P-Nicholas[1], Latha Sridharan[2], Judy Lucas[1], Manoj Charati[2], Andreas Maderna[8], Hans-Peter Gerber[2] Puja Sapra[2] and Lioudmila Tchistiakova[1]
Pfizer Worldwide Research and Development, [1]Global Biotherapeutic Technologies, Oncology Research Unit, [3]ORU Clinical, [4]DSRD, [5]PDM, [6]Precision Medicine, [7]Computional Biology, [8]WWMC, [9]PharmSci, [10]Development Management

NOTCH-antibody drug conjugates have a different mechanism of action than NOTCH signaling inhibitors and induce tumor regression

There are four different NOTCH receptors in mammalian cells that have overlapping patterns of expression in embryonic and adult tissues, but fulfill non-redundant roles during hematopoietic stem cell specification, T cell development, intestinal crypt cell specification and vascular development. NOTCH receptors are over-expressed or amplified in certain human tumors and regulate cell proliferation, differentiation, and survival through an intracellular domain that functions as a transcriptional activator. Several strategies are in development to block NOTCH signaling for therapeutic purposes in cancer, including gamma-secretase inhibitors that block all NOTCH signaling and antibody-based targeting of individual receptors. However, blocking pathway activation with inhibitory antibodies has proven to be less efficacious than originally anticipated. We have generated antibody-drug conjugates (ADCs) that combine the specificity of high affinity anti-NOTCH antibodies with the cytotoxicity of microtubule inhibitors. These ADCs significantly enhance efficacy and also allow targeting in tumors that overexpress NOTCH but are not driven by its signaling. NOTCH-ADCs inhibited the in vitro growth of lung, breast and ovarian cancer cell lines in the low ng/ml range and in vivo regressed the growth of established human tumor xenografts. Our

data demonstrate that NOTCH-ADCs are potent therapeutics capable of inducing sustained tumor regressions in pre-clinical models.

5.35 pm **Agamemnon Epenetos - Adjourn to 2015**

POSTER PRESENTATIONS

These will be displayed in the Hall area during the whole period of the conference

Helen Sheldon[1], Esther Bridges[1], Evelyn Ramberger[2], Esther Kleibuker[3], Massimo Maseiro[4] and Adrian L Harris[1]

[1]Department of Medical Oncology, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom, [2]The University of Applied Sciences, FH Campus Wein, Vienna, Austria, [3]VU University Medical Centre, Amsterdam, The Netherlands, [4]Nuffield Department of Clinical Laboratory Sciences, John Radcliffe Hospital, Oxford, United Kingdom.

RHOQ – an essential mediator of the Dll4/Notch regulation of angiogenesis

Notch signalling is an essential pathway in angiogenesis. The endothelial specific ligand, Dll4 activates the Notch receptor and enables tip cell/stalk differentiation during the early stages of vessel formation. Notch signalling regulates the transcription of a number of target genes and we are interested in identifying novel targets to establish their role in vascular biology and their interactions other angiogenic signalling pathways. Using deep sequence analysis of RNA extracted from Human Umbilical Vein Endothelial Cells (HUVEC) stimulated with Dll4, we identified RHOQ as a gene which is regulated by Notch signalling. RHOQ is a member of the Rho family of small GTPases, which switch between inactive GDP-bound and active GTP-bound states and function as molecular switches in a number of signal transduction pathways. Rho proteins promote reorganization of the actin cytoskeleton and regulate cell shape, attachment, and motility. RHOQ has been shown to play a significant role in insulin-stimulated exocytosis of glucose transporters, possibly acting as the signal that turns on the membrane fusion machinery. It has also been described in neurite outgrowth and membrane expansion in developing neurons. The role of RHOQ is currently unknown in endothelial cells, so to assess its function we have knocked out RHOQ and over-expressed it using lentivirus. Although RHOQ is a Notch target gene it is also appears to be involved in the signalling process. Loss of RHOQ in HUVEC inhibited

the cells ability to signal in response to Dll4. The Notch receptor is still able to cleave but its translocation to the nucleus is impaired. RHOQ co-localises with NICD and EXOC7, a member of the exocyst complex, which plays a critical role in vesicular trafficking targeting post-Golgi vesicles to the plasma membrane. Together these proteins appear to be crucial in trafficking NICD to the nucleus. Over-expression of RHOQ enabled the cells to react quicker to Dll4 stimulation with Notch target genes increasing as early as 2 hours after plating the cells onto the ligand. Consistent with a role in Notch signalling overexpression of RHOQ leads to fewer, longer sprouts whereas loss of RHOQ leads to hyper-sprouting in a hanging drop assay *in vitro*. The cells have increased VEGFR2 activity and the hypersprouting observed can be blocked with sunitinib, a VEGFR2 inhibitor. *In vivo* matrigel plugs impregnated with siRNA targeting RHOQ had a reduced vessel density compared to siControl plugs and lentiviral treatment of vessels in the Chick Chorioallantoic Membrane (CAM) assay also resulted in dysfunctional angiogenesis. These results suggest that RHOQ may have a central role to play in Notch mediated angiogenesis and as such be a potential target in Notch inhibition strategies.

Iris A.K. Lähdeniemi1, Julia O. Misiorek1, Christian J.M. Antila2, Joel H. Nyström1, Lina E. Fortelius1, Cecilia Sahlgren1,2,3, Diana M. Toivola1
1 Cell Biology, Dept. of Biosciences, Åbo Akademi University, Turku, Finland
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3 Technical University of Eindhoven, Eindhoven, The Netherlands

The role of keratins in proliferation and differentiation of colonic epithelial cells

The Notch signaling pathway is a key regulator of colonic epithelial homeostasis, promoting differentiation towards enterocytes rather than enteroendocrine and goblet cells. Knockdown of murine keratin 8 (K8-/-), the major intermediate filament protein of intestinal epithelial cells known to function as stress protectors, leads to an early colitis disease phenotype. This involves colonocyte hyperproliferation, decreased apoptosis and consequently longer colon crypts. Since heterozygote (K8+/-) mice, which express ~50% less keratins compared to K8+/+, have an intermediate hyperproliferation phenotype but no inflammation, a link between keratins and proliferation signaling pathways could be anticipated. The aim of this study is to understand how keratins modulate Notch signaling and

colonic epithelial cell proliferation and differentiation. mRNA levels on the stem cell marker lgr5 are significantly decreased in K8-/- mice and additionally BrdU and phospho-Histone H3 staining indicate that both the amount and migration speed of proliferative cells are increased in K8-/- and K8+/- compared to K8+/+. Interestingly, the amount of goblet cells and enteroendocrine cells are significantly increased while the amount of enterocytes is significantly decreased in K8-/- mice compared to K8+/+ mice. Moreover, the Mucin RT-PCR results indicate that goblet cells do not function properly and are smaller in K8-/- and K8+/- mice. This dramatic change in differentiation pattern in both K8-/- and K8+/- mice colonic epithelium correlates with significantly decreased epithelial levels of both full length and activated Notch, and consequently decreased levels of the key Notch target genes Hey1 and Hey2, in K8-/- mice. Taken together, these results indicate that a decrease in or a lack of keratins disrupts key signals in maintaining instestinal homeostasis of colonic epithelial cells and keratins could thereby regulate both proliferation and differentiation of colonic epithelium.

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Identification of novel regulators of the Notch ligands Delta-Like 1 and 4 as potential therapeutic targets for T-cell Acute Lymphoblastic Leukemia (T-ALL)

Introduction: Notch signaling pathway plays a key role in stem cell self-renewal, cell proliferation, and differentiation. Consequently, it has important developmental functions, and its aberrant activation leads to many diseases and cancers, including T-cell Acute Lymphoblastic Leukemia (T-ALL). Understanding how Notch signaling is regulated is thus central to understand cancer development and to identify new potential therapeutic targets. The Notch receptor responds to transmembrane ligands of the DSL family (Delta/Serrate/Lag-2) and many cancers involving Notch are ligand-dependent. For instance, activating mutations in NOTCH1 have been identified in over 55% of T-ALL, with 40% of these mutations resulting in ligand hypersensitivity or ligand-independent Notch activation. Strikingly, the molecular mechanisms that regulate ligand activation in the signal-sending compartment are yet to be fully characterized. Therefore the aim of this project is to identify regulators of the

ligand Delta activity that subsequently modulate physiological Notch signaling, as well as its oncogenic activity in T-ALL.
Methods and Results: To unravel new Delta regulators, we performed a genome-wide shRNA screen using an in vitro co-culture assay, and targeting specifically the signal-sending cells. We used the OP9 mouse stromal cells stably expressing the ligand Delta-like 1 (DL1) as signal-sending cells, and HeLa cells stably transfected with a luciferase reporter of Notch as signal-receiving cells. This screen allowed us to identify 166 primary hits, which were further confirmed in OP9 cells expressing another DSL ligand: DL4. Using functional secondary assays, we found that those hits are required for Notch-mediated events such as differentiation of hematopoietic progenitors into T-cells, and maintenance of pre-leukemic stem cells (pre-LSCs) isolated from a T-ALL mouse model. Moreover, we showed that some newly identified regulators are necessary for the progression of pre-leukemic T-cells into leukemic T-cells in vivo, following engraftment. This latter experiment demonstrates that pre-LSCs require ligand activation of Notch to induce leukemia, and validates our hits as potential molecular targets for the treatment of T-ALL.
Conclusion and Relevance: Altogether, our screen and the validation experiments allowed us to identify new classes of Notch ligand regulators. These could serve potentially to develop novel therapeutic strategies targeting the stromal microenvironment in Notch cancers, as exemplified by the T-ALL model.