

Project proposal

Molecular mechanisms in normal and diseased gastrointestinal system:

**Focus on neuroendocrine (NE) tumour biology and gastrin
responses in NE tumour cells**

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Introduction

Functional genomics is a new discipline where knowledge gained from whole genome sequencing is exploited in order to gain insight into molecular mechanisms underlying normal biology and disease. Main challenges are to describe the functions of all genes and their products (today, the function of only 6-7.000 of about 20.000 - 25.000 human genes/gene products are known), and to understand the roles of these genes and gene products in normal biology and disease. Central to functional genomics are genome-wide scans of gene expression and proteins, and use of computer science and bioinformatic tools to analysis and modeling the vast amount of data. The results from this research will provide the basis for an array of new medical treatments and diagnostic tools, e.g. presymptomatic cancer diagnosis.

Gastrin - a central gastrointestinal mediator

Gastrin is a central regulator of function and growth of the gastric mucosa and may be involved in growth regulation in gastrointestinal and stem cell-derived tumours. A key to the molecular mechanisms underlying physiological and pathophysiological processes where gastrin may be involved, is the identification of genes that are regulated by gastrin and characterization of the mechanisms involved in gastrin-mediated regulation of gene expression. Neuroendocrine tumours (NETs) constitute a heterogeneous group of neoplasms which may arise in virtually all tissues and organs as a consequence of malignant transformation of various types of NE cells¹. NETs arising in the gastrointestinal (GI) system are by far the most common. Although the incidence is rather low², the prevalence is high, reflecting in general, a high grade of differentiation, slow growth rate, and a low malignancy potential.

There is a need for improved diagnostic tools for prognosis and choice of treatment as well as for early detection of tumours. Better understanding of molecular mechanisms of tumour biology and access to a wider range of informative molecular markers will be mandatory for such diagnostic tools.

Gastrin is mainly produced by G-cells in the antrum mucosa in response to food and is under negative feedback regulation by gastric acid. Thus, gastrin levels in the normal individual fluctuate with the meals during of the day. Gastrin activates acid secretion by endocrine stimulation of enterochromaffin-like cells (ECL) which secrete histamine and thereby activate H⁺-secretion from parietal cells in oxyntic mucosa³. Gastrin also stimulates growth of oxyntic mucosa and ECL cells *in vivo*⁴ and in pathological conditions that lead to hypergastrinemia in man⁵. Moreover, it has been suggested that gastrin is involved in autocrine growth regulation in gastrointestinal and stem cell derived tumours⁶.

Many genes that are transcriptionally activated by gastrin contain cAMP responsive promoter elements (CRE). However, it was not known whether gastrin could regulate gene expression via these promoter elements. We have shown that gastrin can induce gene expression via CRE⁷. The promoter element CRE was first identified as the element responsible for cAMP mediated activation of the somatostatin gene⁸, and has been found to be involved in transcriptional regulation of a number of other genes like glucagon, insulin, gastric inhibitory polypeptide, calcitonin gene-related peptide and neurotensin⁹. CRE-promoter elements are also central in

¹ Öberg K, 2000. *Quart. J Nucl Med* 44, 3-12

² Hestad K, Falkmer S, Falkmer U. 2001. *MD student thesis*, Faculty of Medicine, NTNU, Trondheim

³ Sandvik AK, Waldum HL, Kleveland PM, Schulze Søgne B, *Scand J Gastroenterol*, 22:803, 1987

⁴ Brenna E, Waldum H, *Gut*, 33:2129, 1992

⁵ Mattsson H, Havu N et al, *Gastroenterology* 100:311, 1991; Havu N, Maaroos H-I, Sipponen P, *Scand J Gastroenterol*, 26:90, 1991

⁶ Baldwin GS. *J Gastroenterol Hepatol* 10:215, 1995; Schaer JC, Reubi JC, *J Clin Endocrinol Metab*, 84:233, 1999

⁷ Thommesen L, Hofslø E, Paulssen R, Anthonen MW, Lægreid A. *Am J Physiol* 281: E1316, 2001

⁸ Montminy MR et al *Proc Natl Acad Sci USA* 83:6682, 1986

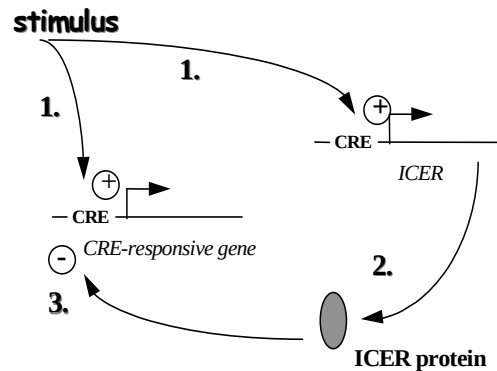
⁹ Habener JF et al *Vit Horm* 51:1, 1995; Someya Y et al *FEBS Lett* 317:67, 1993; Watson A, Latchman D *J Biol Chem* 270:9655, 1995; Evers BM et al *Mol Cell Biol* 15:3870, 1995

regulating the proto-oncogene c-fos, as shown in proliferation of pituitary somatotroph cells and in regulation of growth and differentiation of the pheochromocytoma cell line PC12 by nerve growth factor¹⁰. Thus, CRE-mediated gene regulation is of central interest in studies of molecular mechanisms underlying regulation of function and growth of neuroendocrine cells.

ICER - a putative tumour suppressor involved in gastrin-mediated gene regulation

Our observation that gastrin activates transcription of the transcriptional repressor ICER (inducible cAMP early response gene)¹¹ is highly interesting as it indicates that ICER may participate in termination of gastrin induced transcription via CRE-promoter elements.

ICER is a repressor belonging to the CREB-family of transcription factors. ICER is activated via cAMP responsive promoter elements (CRE)¹² and is known to inhibit CRE-regulated gene expression¹³. It has been suggested that ICER may be involved in negative feed back regulation of physiological processes like diurnal rhythm regulated production of melatonin in the pineal gland¹⁴



A number of studies indicate that ICER is involved in regulating cell growth. ICER is induced during liver regeneration after subtotal hepatectomy¹⁵, and has been shown to be involved in proliferation of thyroid cells induced by thyroid-stimulating hormone¹⁶. In the leukemia-derived cell line IPC-81, cAMP-induced apoptosis is inhibited by ICER overexpression, indicating that ICER may block genes involved in induction of apoptosis¹⁷. In three different tumour cell lines: the choriocarcinoma cell line JEG, the pituitary-derived cell line At20, and in the prostate cell line LNCaP, overexpression of ICER leads to inhibition of growth and reduced tumourigenesis in nude mice¹⁸. This indicates that ICER may act as a tumour suppressor in several different cell types. ICER and CREB have recently been suggested to play a role in tumourigenesis of neuroendocrine tissues¹⁹. We have recently found that endogenously expressed ICER suppresses gastrin-mediated transcriptional activation of c-fos²⁰.

Alternative splicing of ICER (γ and DBD exons) generates ICER I, I γ , II and II γ , and raises the possibility that ICER splice variants may differ in their modes of action as repressors. Variation of the splicing process may occur during tumour progression and probably play an important role in carcinogenesis²¹. Increased ICER I γ expression in β cells resulted in early severe diabetes²², while ICER II γ evokes neuronal apoptosis²³ and has also been reported to inhibit the growth of pituitary tumour cells and choriocarcinoma cells¹⁶. However, in none of these studies the effect of two or more splice variants was compared.

¹⁰ Sassone-Corsi P *et al* *Genes Dev*, 2:1529, 1988; Ginty DD *et al* *Cell*, 77:713, 1994

¹¹ Thommesen L, Nørsett K, Hofslø E, Sandvik AK, Lægneid A. *J Biol Chem*, 2000, 275:4244

¹² Sassone-Corsi P *et al*, 1995, *Annu.Rev.Cell.Dev.Biol* 11, 355-377

¹³ Molina, CA *et al*, *Cell*, 75:875, 1993

¹⁴ Stehle HW *et al*, *Naturwissenschaften*, 83:535, 1996

¹⁵ Servillo *et al*, *Oncogene*, 14: 1601, 1997

¹⁶ Uyttersprot N *et al*, *Eur J Biochem*, 1999, 259:370.

¹⁷ Ruchaud, *Oncogene*, 15: 827, 1997

¹⁸ Razavi *et al*, *Oncogene*, 17: 3015, 1998, Yehia *et al*, *Cancer Research*, 61: 6055, 2001

¹⁹ Rosenberg D *et al*, *Ann NY Acad Sci* 968: 65, 2002

²⁰ Steigedal TS, Bruland T, Misund K, Thommesen L, Lægneid A. Inducible cAMP Early Repressor (ICER) Suppresses Gastrin-Mediated Activation of cyclin D1 and c-fos gene expression (manus in prep)

²¹ Brinkman BM. *Clin Biochem* 2004; 37(7), 584-594.

²² Inada A, Someya Y, Yamada Y, Ihara Y, Kubota A, Ban N, Watanabe R, Tsuda K, Seino Y. *J Biol Chem* 1999; (30) 21095-21103.

²³ Jaworski J, Mioduszevska B, Sanchez-Capelo A, Figoel I, Habas A, Gozdz A, Proszynski T, Hetman L, Mallet J, Kaczmarek L. 2003 *J neurosci*. 23 (11), 4519 – 4526.

Gastrin-mediated signal transduction

Cellular effects of gastrin are transmitted via a specific transmembrane Gq/G11-protein coupled receptor, the CCK-B/ gastrin receptor which targets a cascade of intracellular mediators including phospholipase C- β (PLC- β) and protein kinase C (PKC) as well as activation of Shc, Grb and Sos, Ras and Raf proteins, and mitogen-activated protein kinases (MAPK)²⁴. We have shown that the signal transduction mechanisms for gastrin-induced transcriptional activation of CRE-driven promoters involve PKA, PKC and CAMkinase, but not the MAP-kinase cascade which is known to play a role in gastrin-induced cell proliferation²⁵. Only few gastrin-responsive genes have been reported in the literature. Using our 2,500 probe microarrays, we have identified 250 gastrin-responsive genes in the human carcinoid cell line BON. Many of these genes are involved in cellular proliferation, adhesion, motility and in oncogenesis²⁶.

PRINCIPLE OBJECTIVE

The principal objective is to achieve a better understanding of molecular mechanisms involved in gastrin-mediated regulation of cellular, physiological and pathophysiological processes in the gastrointestinal tract; to explore the role of gastrin in NE cells and exploit this knowledge in development of improved diagnostic tools for NE tumours.

Component objectives

- Identify molecular mechanisms involved in gastrin responses
- Identify gastrin-induced genes that are turned off via ICER
- Investigate the role of ICER splice variants in modulation of gastrin-induced responses like gene expression and proliferation of neuroendocrine gastrointestinal cells
- Discover informative molecular markers for NE cancer
- Identify molecular mechanisms involved in NE cancer with the aim to assist in the search for improved treatment

METHODS

Cell line model systems and molecular cell biology methods. We use our established neuroendocrine cell lines as model system for gastrin-induced molecular responses in neuroendocrine cells: one GI carcinoid (BON), two neuroblastomas (SK-N-AS/ SK-N-FI 142), two lung carcinoids (NCI-H727/ UMC-11) and one medullary thyroid carcinoma (TT).AR42J has gastrin/CCK2 receptors²⁷ and expresses the neuroendocrine marker chromogranin A (CgA)²⁸. AGS cells are stable transfected with CCK2 receptor²⁹. Reporter gene cell systems for studies of signal transduction pathways involved in gastrin-mediated activation of chromogranin A-, c-fos- and ICER-promoter have been established. Western blot analysis, immunohistochemistry and BrdU-based cell proliferation methods are established^{27,29,30}. Inducible expression of ICER I and ICER II γ is established in Flp-In HEK 293 cells³⁰(InVitrogen). RNAi knock-down of ICER has been established³¹.

²⁴ Kowalski-Chauvel A, et al, *J Biol Chem*, 271:26356, 1996; Dabrowski A et al, *Digestion* 58: 361, 1997

²⁵ Thommesen L, Hofslis E, Paulsen R, Anthonsen MW, Lægreid A. *Am J Physiol* 281: E1316, 2001

²⁶ Hofslis E, Thommesen L, Yadetie F, Langaas M, Sandvik A, Lægreid A. Identification of novel growth factor-responsive genes in neuroendocrine gastrointestinal tumour cells. *Br. J Cancer* 2005 Apr 25;92(8):1506-16.

²⁷ Christophe J, *Am J Physiol*, 266: G963, 1994

²⁸ Hofslis E, Thommesen L, Nørsett K, Falkmer S, Syversen U, Sandvik AK, and Lægreid A. Expression of chromogranin A and somatostatin receptors pancreatic AR42J cells. *Mol Cell Endocrinol.* 194 (1-2):165-73, 2002

²⁹ Hocker M, Zang Z, merchant JL, Wang TC. Gastrin regulates the human histidine decarboxylase promoter through an AP-1-dependent mechanism. *Am J Physiol.* 1997 Apr;272(4 Pt 1):G822-30

³⁰ Misund K. Hovedfagsoppgave i molekylær biologi, NTNU 2002.

³¹ Tømmerdal LF, Strømme T. Hemming ac CREM genprodukt ved RNA interference. Prosjektoppgave NTNU, 2002.

DNA microarray technology enables expression analysis of thousands of genes in a single experiment³². Printed oligomicroarrays for gene expression analysis in cell lines will be used in collaboration within the Norwegian Microarray Consortium (<http://www.mikromatrise.no>). Oligonucleotide microarrays for specific detection of CREB/CREM/ATF-splice variants has been established³³. The microarray analyses will be verified by RT-PCR and Northern blot as in previous studies^{22, 29}. Splice variants will be measured specifically by RT-PCR and by oligonucleotide microarrays. These analyses will be followed up by measurement of splice variants at the protein level (Western blot, immune precipitation, 2D gel electrophoresis-MS) in collaboration with prof. Geir Slupphaug, NTNU.

Tissue microarray (TMA). In brief, TMA allows for high-throughput molecular profiling of tumour specimens³⁴. The preparations of the TMAs will be done in collaboration with professor Duan Chen and senior researcher Chun-Mei Zhao, who more recently have established the TMA technique in Trondheim.

Cell arrays and revers transfection. High throughput methods for gene hypothesis testing are under establishment in some leading laboratories in functional genomics. One method is by use of transfected cell microarrays. The expression plasmids for genes of interest or siRNA/shRNA are mixed and spotted on glass slides to form an array of nucleic acids ready for transfection. The arrays were placed in tissue culture dishes and culture cells were added to the arrays. Cells adhere to the spots undergo reverse transfection. Subsequent the alteration in cell function can then be detected by use of GFP or other fluorescent-linked proteins. These analyses will be performed in collaboration with post.doc. Torunn Bruland.

In situ hybridization will be used to detect specific transcripts in tissue sections. Methods for detection of ICER and of CREM isoforms of transcriptional regulators are established in the laboratory of our collaborator Dr. Garry Wong, University of Kuopio, Finland³⁵.

PhD project I

Identify the role of ICER splice variants in modulation of gastrin-mediated gene expression

ICER is a product of the CRE-modulator (CREM) gene which encodes 20-25 different basic leucine zipper (bZip) CRE-binding proteins (repressors and activators), and belongs to the CREB/CREM/ATF-family³⁶. ICER is transcribed from an inducible P2 promoter, and is the only factor known to be activated by induction of gene expression. The emerging picture is that the transcriptional regulation of cAMP-responsive genes results from a finely tuned balance of multiple activators and repressors of CRE-regulated promoters.

The aim of the present subproject is to identify the role of ICER in modulation of gastrin-induced gene expression. We want to characterize in detail how the different splice variants are affected by gastrin.

We have shown that gastrin induces the transcriptional repressor ICER in AR42J cells in a rapid and transient manner (max after 2 hours, downregulated after 6-8 hours)³⁷. ICER can function as a negative feedback-regulator of CRE-mediated gene expression (see Figure, page 2). If ICER plays such a role in gastrin responses, we would expect ICER to downregulate gastrin-induced, CRE-regulated genes at 2-10 h after onset of gastrin stimulation. To test this hypothesis we will compare the transcriptional program (microarray analyses) in inducible Flp-In HEK cells overexpression different ICER splice variants.

a) with high constitutive levels of ICER. Genes which are upregulated in cells with control constructs but not upregulated in cells overexpressing ICER are candidates for genes where ICER can function as a negative feedback regulator

³² Schena M, Shalon D, Davis RW, Brown PO, *Science* 270: 467, 1995

³³ Ween O, Establishment of splice variants microarray (manus to be submitted)

³⁴ Kononen J, Bubendorf L, Kallioniemi A et al. 1998. *Nature Medicine* 4, 844-847

³⁵ Storvik M, Lindén AM, Kontkanen O, Lakso M, Castrén E, Wong G, *J Pharm Exp Ther*, 294: 52, 2000

³⁶ Lalli, E et al, *J Biol.Chem.* 268, 17359, 1995

³⁷ Thommesen L, Nørsett K, Hofsløi E, Sandvik AK, Lægreid A. *J Biol Chem*, 2000, 275:4244

b) unable to express ICER (inhibition by RNAi^{46,47}). Gastrin-induced genes that are first induced and then turned off again at 2-10 hours after onset of gastrin stimulation in the wild type but not in the ICER-deficient cells, are candidates for genes where ICER can function as a negative feedback regulator.

Results from these studies will enable us to put forward hypotheses on the role of ICER in gastrin-mediated responses in neuroendocrine cells. In particular, we will seek to identify the biological functions of gastrin-regulated genes where ICER functions as a negative feedback regulator. This will be done by using existing biological background information from public databases and from the literature. One interesting question will be which fraction of these genes are predominantly involved in cellular processes like proliferation and regulation of cell cycle. Another highly interesting follow-up of these studies will be to analyse the promoter regions of the candidate genes identified in the above studies with the purpose to identify common promoter motifs that may be involved in ICER modulation of gene expression. Such promoter motif analyses will initially be focused on CRE-like sequences, but approaches will also be employed that may identify any common motifs. This work will be done in collaboration with prof. Finn Drabløs, NTNU. The hypotheses will be tested by experimental molecular cellular methods in cell line model systems.

We have shown that the signal transduction mechanisms for gastrin-induced transcriptional activation of CRE-promoter elements involve PKA, PKC and CAMkinase, but not the MAP-kinase cascade which is known to play a role in gastrin-induced cell proliferation³⁸. These observations indicate that gastrin can activate a number of different intracellular signaling pathways and that the different pathways may activate distinct sets of genes. In order to obtain insight into the molecular mechanisms involved in gastrin responses, it is of interest to identify the genes that are regulated downstream of each of these pathways.

In these studies we will focus on comparing the gastrin-mediated genome-wide transcriptional program in cells where signaling components like PLC- β , PKA, specific PKC isoenzymes, and MAP kinases are inhibited. Since MAP kinases are involved in gastrin-mediated cell proliferation but not in regulation of CRE-driven promoters⁵⁷, while PKA is important for activation of CRE-promoters⁵⁷, it will be of particular interest to identify the differences in gastrin-induced changes in gene expression in cells where PKA has been inhibited with the gastrin-induced changes in gene expression in cells where specific MAP kinases have been inhibited. The effect of PKA- and MAP kinase-inhibition on gastrin-induced cell proliferation will be measured in the same experimental systems.

PhD project II

Characterization of gene expression in NE and non-NE tumour cells

The management of GI neuroendocrine tumours (NETs) is a great challenge since some patients live for several years, sometimes with debilitating symptoms due to hormone hypersecretion, while others have highly aggressive tumours leading to early death. Currently, little is known about which factors that may predict prognosis or response to therapy.

Somatostatin (SST) analogs play an important role in the treatment of NETs, and NETs characteristically show a high expression of SST receptors (SSTRs)³⁹. In addition to SST analogs, surgery, alpha-interferon and chemotherapy are well-established treatment modalities⁴⁰. However, only surgery has the potential of being curative. Thus, new treatment modalities such as targeting therapy are warranted.

³⁸ Thommesen L, Hofslø E, Paulsen R, Anthonsen MW, Lægrend A. *Am J Physiol* 281: E1316, 2001

³⁹ Öberg K *Quart. J Nucl Med* 44, 3, 2000; Hofslø E. *Tidsskrift Nor Lægeforening*, 122,487, 2002

⁴⁰ Öberg K, *Ann Oncol* 12, S111, 2001

As mentioned above, in three different tumour cell lines, overexpression of ICER leads to inhibition of growth and reduced tumourigenesis in nude mice⁴¹. This indicates that ICER may act as a tumour suppressor in several different cell types. Furthermore, ICER and CREB have recently been suggested to play a role in tumourigenesis of neuroendocrine tissues⁴².

Alternative splicing of ICER raises the possibility that ICER splice variants may differ in their modes of action as repressors. Variation of the splicing process may occur during tumour progression and probably play an important role in carcinogenesis⁴³. Increased expression of ICER II γ has been reported to inhibit the growth of pituitary tumour cells and choriocarcinoma cells¹⁶.

More recently we have, by cDNA microarray analysis of various non-NE and NE tumour cell lines, identified novel NET-specific genes (Hofsli, to be submitted), which may serve as new diagnostic NE markers.

One aim of this subproject is to characterize the expression of the various ICER/CREM splice variants in NE tumour cells and compare the pattern to that in non-NE tumour cells. We will do this by using our established NE and non-NE cell lines as a model system.

Results from these studies will enable us to put forward hypotheses on the role of ICER and the various splice variants in NE tumour biology as compared to non-NE tumour biology. If different patterns are found, this may indicate that ICER plays an important role in NE tumourigenesis as compared to the situation in other solid tumours.

Furthermore, the results of the cell line studies will be tested by studying tumour samples from patients with GI NE tumours. This will be done by in situ hybridization analysis of various ICER/CREM transcripts in paraffin-embedded tumour samples from 55 ileum carcinoid patients (already collected at St. Olavs University Hospital). Methods for detection of ICER and of CREM isoforms of transcriptional regulators are established in the laboratory of our collaborator Dr. Garry Wong, University of Kuopio, Finland⁴⁴.

Our cDNA microarray analysis of various non-NE and NE tumour cell lines has also identified genes that are strongly underexpressed in NE tumour cells compared to non-NE tumour cells. Therefore, another aim of this project will be to investigate whether these genes may represent tumour suppressors in the context of NE tumour biology. One mechanism of gene silencing is hypermethylation of gene promoters, and this will be investigated by methylation-specific RT-PCR analysis.

Finally, by studying gastrin-induced gene and protein expression in NE and non-NE tumour cells, we hopefully will give more information to the question whether gastrin may play a role in tumourigenesis in both NE and non-NE tumours. As we previously has shown that the somatostatin analog octreotide inhibits gastrin-induced proliferation in AR42J cells, we will also compare gene/protein expression in NE vs. non-NE cells after treatment with octreotide.

Time table

2006: Stimulation of ICER I /II γ overexpression cells, microarray analysis. Put forward hypotheses about the role of ICER splice variants in these cells (project 1). Study the expression of ICER splice variants in NE and non-NE cell lines (project 2). Summerize putative common hypotheses and further experiments (both projects).

⁴¹ Razavi *et al*, *Oncogene*, 17: 3015, 1998, Yehia *et al*, *Cancer Research*, 61: 6055, 2001

⁴² Rosenberg D *et al*, *Ann NY Acad Sci* 968: 65, 2002

⁴³ Brinkman BM. *Clin Biochem* 2004; 37(7), 584-594.

⁴⁴ Storvik M, Lindén AM, Kontkanen O, Lakso M, Castrén E, Wong G, *J Pharm Exp Ther*, 294: 52, 2000

- 2007:** Use of chemical inhibitors and/or siRNA to elucidate intracellular signaling pathways involved in gastrin-mediated gene responses in cells overexpression ICER splice variants (project 1). Further characterize ICER/CREM splice variants in NE versus non NE tumours, to explore possible splice variants important i tumourigenesis (project 2). In situ hybridization of various ICER/CREM products in tumours from carcinoid patients (project 2).
- 2008:** Use of siRNA towards genes found to be regulated by ICER. Study the effect of gastrin -mediated (and other growth factors) gene responses in cells where these genes are knocked down (project 1). Study gastrin-responses in NE and non-NE tumour cells (project 2).
- 2009:** Study the effect of siRNA knock down of PKC and PKA signaling pathways (project 1). Compare gene/protein expression in NE versus non-NE cells after octreotid treatment (project 2).
- 2010:** Complete PhD thesis (project 1 and 2)

Collaborators:

- Assoc. Prof. Mette Langaas, PhD. Statistic and mathematics, IKM, NTNU
- Prof. Arne Sandvik, MD, PhD. Molecular Physiology, clinical gastroenterology, NTNU/St.Olav
- Post.doc. Endre Anderssen, PhD. Bioinformatics, modelling, statistic, NT, NTNU
- Prof. Finn Drabløs, PhD. Bioinformatics, DMF, NTNU
- Prof. Marit Otterlei, PhD. Confocal microscope/ read-outs IKM, NTNU
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- Prof. Duan Chen, MD, PhD. Experimental Surgery, LKB, NTNU
- Assoc. Prof. Chun-Mei Zhao, PhD. Molecular Biology, LKB, NTNU