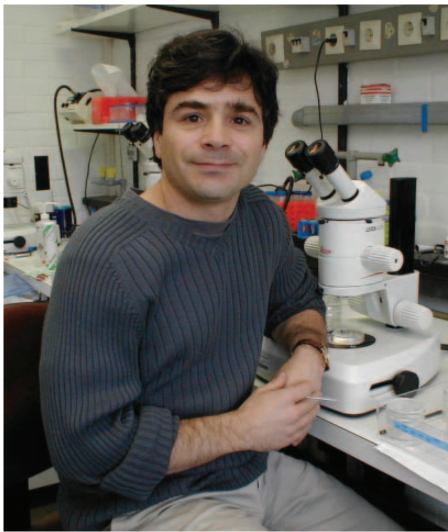
Independent Junior Research Groups - Otto-Warburg-Laboratory

The Otto-Warburg-Laboratory consists of three independent Junior Research Groups, headed by Adam Antebi, Ann Ehrenhofer-Murray, and Andrea Vortkamp.

Endocrine regulation of *C. elegans* development & aging



Head:
Dr. Adam Antebi
Phone: +49 (0)30-8413 1302
Fax: +49 (0)30-8413 1130
Email: antebi@molgen.mpg.de

Scientist:
Dr. Birgit Gerisch

Graduate students:
Andreas Ludewig (Ph.D. 4/2003)
Nicole Fielenbach
Veerle Rottiers
Axel Bethke

Diploma students:
Daniela Gibis
Nanyi Park

Undergraduate student:
Phillip Thoman

Technicians:
Cindy Weitzel
Kerstin Neubert
Anne Frenzel

Visiting scientists/students:
Dr. Paul Dowell (Johns Hopkins U.)
Neal Freedman (UCSF)

Summary

All animals develop through successive stages and have defined life spans determined by their genome and modulated by environment. What are the underlying molecular mechanisms that specify life stages and influence the pace of aging? Using *C. elegans* as a genetic model, my laboratory has discovered that a nuclear receptor signaling cascade works downstream or parallel to insulin/IGF signaling to influence life histo-ry decisions. Our work provides powerful insights into how nuclear receptor signaling pathways behave in the context of organismal endocrine networks, with medical relevance to our understanding of diabetes, obesity and aging.

Background

A striking finding is that single-gene mutations can transform the identity of *C. elegans* life stages and regulate life span. A handful of genes, the heterochronic loci, act as "stage selectors" or master regulators of stage-specific temporal fates. They encode diverse transcriptional and translational regulators, many of them conserved. Another set of genes, the dauer loci, regulate a "developmental checkpoint", the choice between reproductive development and developmental arrest at an alternate third larval stage, the dauer diapause, in response to starvation cues.

Dauer larvae store fat, are stress resistant and long-lived. Identified signaling pathways, including insulin/IGF and TGF-β, act by a neuroendocrine mechanism to regulate the dauer decision (Figure 1). Interestingly, it has been shown that insulin/IGF signaling also influences adult longevity. For example, mutations in the *daf-2*/insulin/IGF receptor (InsR) double adult life span. Remarkably, recent work in flies and mice suggests that reduced insulin/IGF signaling similarly affects life span, revealing that this constitutes an ancient mechanism to promote survival under stress. Evidence suggests that insulin/IGF signaling regulates diapause and life span cell-nonautonomously by a downstream hormonal signal. We have likely found part of this hormonal signal with the discovery of nuclear hormone receptor, *daf-12* and functionally related genes, which act at the nexus of pathways regulating temporal identity, diapause and life span.

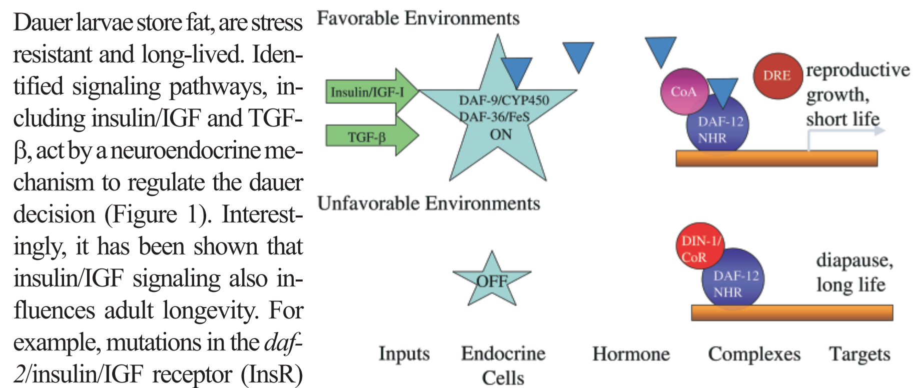


Figure 1: Endocrine regulation of C. elegans life history

Results

DAF-12 nuclear receptor

We originally discovered that *daf-12* links dauer and heterochronic pathways, coupling environmental cues to developmental timing circuits. In collaboration with Don Riddle (U. Missouri) we showed that *daf-12* encodes a nuclear hormone receptor (nhr), transcription factors responsive to lipophilic hormones such as steroids and retinoids. DAF-12 is most similar to vertebrate vitamin D, pregnane, and androstane receptors. This molecular identity predicts life history regulation by lipophilic hormones.

A hormone metabolic pathway

Consistent with this prediction, we found that *daf-9*, a locus with phenotypes similar to *daf-12*, encodes a cytochrome P450 (cyp450) related to vertebrate steroidogenic and fatty acid hydroxylases, suggesting a function in the metabolism of a DAF-12 hormone (Figure 1). Interestingly, strong *daf-9* mutants are modestly long-lived, and weak alleles enhance *daf-2*/InsR longevity to fourfold. In addition, *daf-9(+)* and *daf-12(+)* are required for the longevity of animals whose germline has been removed. Importantly, the molecular identities of *daf-9* and *daf-12* together provide the first strong evidence of lipophilic hormone signaling in *C. elegans*, implying that such hormones modulate life plans and life spans. A sterol derivative may be the hormone, since cholesterol deprivation phenocopies *daf-9* defects (Birgit Gerisch, Veerle Rottiers).

Another locus, *daf-36* phenotypically resembles *daf-9*, and likely defines part of the same pathway. Indeed, we have recently molecularly identified *daf-36* and shown that it encodes an Rieske type FeS dioxygenase most related to bacterial steroid metabolizing enzymes, consistent with a role in hormone metabolism (Veerle Rottiers).

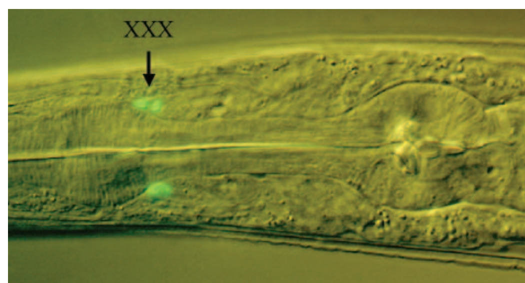


Figure 2: daf-9 ist expressed in XXX endocrine cells.

Hormonal regulation

Importantly, *daf-9* expressing cells identify novel nematode endocrine tissues, and include hypodermis, spermatheca, and a mysterious pair of neuron-like cells, aptly named XXX (Figure 2). We have shown that *daf-9* acts cell non-autonomously and is feedback regulated by *daf-12* itself, consistent with a hormonal mechanism. More-

over, *daf-9* is tightly regulated by nutritional cues and genetic inputs, demonstrating that it is a central point of control. Finally, *daf-9* overexpression rescues larval defects of *daf-2*/insulin receptor mutants, suggesting that sterol hormones work downstream of insulin signal transduction. *daf-36* is expressed in different tissues than *daf-9* (intestine, a few neurons) showing that hormone biosynthesis is distributed (Birgit Gerisch, Veerle Rottiers).

DAF-12 transcriptional complexes

In the presence of hormone, nuclear receptors typically assemble coactivator complexes that turn on transcription, whereas in the absence of hormone they assemble corepressor complexes that turn off the same targets. Through yeast two-hybrid screens, we identified *din-1*, a homolog of the human SHARP transcriptional corepressor. We propose that *din-1* comprises part of a hormone regulated binary switch that together with apo-DAF-12 implements developmental arrest, diapause and long life (Figure 1). Although nuclear receptors are typically known for hormone activation, our work points to the diametric possibility that repressor function is central to animal development (Andreas Ludewig, Axel Bethke).

New heterochronic genes

In mutant screens for enhanced gonadal heterochrony of *daf-12* null alleles we found two loci, *dre-1* and *dre-2*. *dre-1* displays heterochronic phenotypes in epidermal seam cells as well as in gonad. In double mutant combinations with other heterochronic loci, *dre-1* reveals previously unseen roles in the gonad, opening up gonadal heterochrony to systematic investigation. Finally, *dre-1* affects molting, suggesting a functional link between two developmental timers, the molt cycle, and the heterochronic circuit. It encodes a highly conserved protein that contains F-box, Zinc finger (Zf_UBR-1) and carbohydrate binding domains, implying a role in ubiquitin mediated proteolysis (Nicole Fielenbach).

Target genes

Given *daf-12´s* multiple functions, it is vital to identify target genes. In collaboration with Keith Yamamoto (UCSF) a *daf-12* binding site has been defined. Now we are examining physiological targets such as *daf-9* and other genes using a bioinformatic approach, to test whether transcription is affected (Axel Bethke).

Other aging genes

RNAi by feeding affords a high throughput method for analyzing gene function. We are systematically screening for candidates that give long-lived phenotypes, as well as performing enhancer and suppressor screens with dauer pathway mutants. After having screened through genes on Chr I we have found that among other things, reduced mitochondrial gene function extends life span. In addition, many of these same genes apparently enhance dauer formation (Gudrun Peiler, Nanyi Park, Daniela Gibis).

Significance and future plans

My goal is to understand how identified endocrine signaling pathways, as well as new components regulate life stage programs and life spans. In the short term, I will continue work on the DAF-12 hormone signaling pathway in a broad sense, including dissecting endocrine inputs and transcriptional outputs.

1. We wish to understand more precisely how insulin/IGF and nuclear receptor signaling are coupled, since they show both epistatic and synergistic interactions. Are they connected through kinase cascades, transcriptional control or other ways? Such studies might shed light on disease states where insulin and nhr signaling converge, e.g. diabetes, ischemia.

2. Identification of *daf-9* and *daf-36* expressing tissues has opened up the field of worm endocrinology. Subsequently, several labs have found that Niemann-Pick C1 homologs, which mediate sterol transport, are expressed in the same cells and function in dauer formation. Cholesterol transport and metabolism are now intensely studied, given the connection to hormone production, as well as the medical relevance to age related diseases, such as arteriosclerosis and inflammation.

3. Understanding how nuclear coactivators and corepressors modulate nhr activity in this context may reveal how different life stages and life spans are specified. Genetic screens to identify further corepressor and coactivator components are underway, as are biochemical/proteomic approaches to identify transcriptional complexes, so that we can better study events at the molecular level.

4. A current challenge is to now biochemically identify the DAF-12 ligand, as well as other putative hormones. We have initiated several collaborations to see if *daf-9*, *daf-12* and other mutants have altered sterol profiles. In addition, we have begun pilot screens using sterol compounds to look for biological activity.

5. We will continue the hunt for *daf-12* target genes, both by characterizing specific candidates to connect genetic circuits, as well as whole genome microarray approaches to illuminate new physiological roles. Target genes will also provide an important tool to assay the activity of a DAF-12 ligand.

6. We will also continue exploratory systematic genome wide RNAi screens for other genes influencing longevity, diapause and developmental timing. High throughput methods will be developed so that we can rapidly run whole genome scans by RNAi feeding in various genotypes.

7. In the long term, I am excited to find out if functional vertebrate homologs of *daf-12* and related genes work analogously to affect metabolism, developmental timing, and life span. It is well known for example, that the timing of mammalian puberty is contingent upon appropriate nutritional signals. At the molecular level, insulin/IGF, estrogen receptors and other related molecules may mediate some of these effects. Whether or how these pathways influence life span is not yet known.

General information

Publications 1998-2003

Tatar M, Bartke A & **Antebi A** (2003). *Endocrine Regulation of Aging by Insulin-like signals*. Science 299:1346-1351

Gerisch B & **Antebi A**. *Hormonal signals produced by DAF-9/cytochrome P450 regulate C. elegans dauer diapause in response to environmental cues* (submitted)

Ludewig A, Kober-Eisermann C, Weitzel C, Neubert K, Bethke A, Gerisch B, Hutter H & **Antebi A.** *A complex of nuclear corepressor DIN-1 and nuclear receptor DAF-12 specify C. elegans dauer diapause* (submitted)

Shostak Y, Van Gilst MR, **Antebi A** & Yamamoto K. *In Vitro Genomic Selection: From C. elegans DAF-12 Binding Sites To Target Gene* (submitted)

Gerisch B, Weitzel C, Kober-Eisermann C, Rottiers V & Antebi A (2001). *A hormonal signaling pathway influencing C. elegans metabolism, reproductive development and life span.* Dev. Cell 1: 841-851 (featured article on Science SAGE/ke website, News Focus Dec. 12, 2001)

Antebi A, Yeh WH, **Tait D**, Hedgecock EM & Riddle D (2000). *daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in C. elegans*. Genes & Dev 14: 1512-1527

Antebi A, Culotti JG & Hedgecock EM (1998). *daf-12 regulates developmental age and the dauer alternative in C. elegans.* Development 125: 1191-1205

Invited talks at meetings and symposia 1998-2003

Jacques Monod Conference, Form and Function in Development and Disease, La Londe-les-Maures (6/2003)

The Biology of Human Aging, Brown University, Providence (5/2993)

Gordon Research Conference on Biology of Aging, Ventura (3/2003)

Senior Seminar on Aging, Swarthmore College, Swarthmore (9/2002)

Buck Symposium on Neuroendocrine Systems and Life Span Determination, Novato (9/2002)

European *C.elegans* Meeting, Blankenberge (5/2000)

Keystone Symposium on Nuclear Receptors, Steamboat Springs (3/2000)

Japanese Worm Meeting, Kanazawa (7/1998)

European Worm Meeting, Cambridge (6/1998)

Teaching

C. elegans practical course, Freie Universität, Berlin (5/2003, 3 weeks)

Invited lecturer, Universität Göttingen, Göttingen (7/2002)

Invited lecturer, Dept. of Biochemistry and Ecotoxicology, Freie Universität, Berlin (4/2002)

Invited lecturer, Dept. of Parasitology, Humboldt-Universität, Berlin (2/2002)

Invited lecturer, University of Ghent, Ghent (9/1998)

Theses

Andreas Ludewig: *Nuclear receptor pathways in C. elegans: DIN-1, a DAF-12 coregulator of dauer diapause and developmental arrest*. PhD Thesis at Freie Universität Berlin (4/2003, MPG and EC Agegen grants)

Appointments, scientific honors & memberships

Participant of Science SAGEKE website (2002-present)

Member of the German Genetics Society (1999-2000)

External funding

QLK6-CT-1999-02071, European Community Grant, Agegen: *The identification and characterisation of effector genes in the C. elegans enhanced life maintenance program* (2000-2003)

Institute collaborations

Functional genomic analysis of Chromosome 21 homologs in C. elegans, with Marie Laure Yaspo, Dept. Lehrach

Molecular evolutionary studies on C. elegans runt homolog, with Volkhard Seitz, Research Group Mundlos

Transcriptional complexes in dauer formation, with Johan Gobom, Dept. Lehrach

Bioinformatic discovery of DAF-12 target genes, with Christoph Dieterich, Dept. Vingron

Consulting activities

Scientific Advisory Board, Wellcome Trust Grant on Functional Genomics of Aging (2002-07)

Consultant, Devgen Corporation (1997-98)

Institute activities

initiated Institute-wide seminar series and chair of seminar committee (5/2000-present)

served on MPIMG election committee (2/2002-present)

served on steering committee for Institute Day of Scientific Exchange (2/2002)

Public relations

Featured scientist on Max Planck Forum on Aging, Deutsche Welle TV (5/2003)

Featured scientist on Science´s SAGEKE website (4/2003)

Berlin Long Night of Sciences, open house visit and presentation of the laboratory to the public (6/2002)

Featured scientist on Swedish National Radio (2/2002)

Featured scientist in television production on aging, channel ZDF (4/2000)

Gene silencing in *Saccharomyces cerevisiae*

Head:
PD Dr. Ann Elizabeth Ehrenhofer-Murray

Phone: +49 (0)30-8413 1329
Fax: +49 (0)30-8413 1130
Email: ehrenhof@molgen.mpg.de

Scientists:
Dr. Jacqueline Franke (since 6/02)
Dr. Sigrid Schaper (since 7/01)
Dr. Arnold Grünweller (until 1/02)

Graduate students:
Stefan Ehrentraut (since 8/03)
Antje Geissenhöner
Horst Irlbacher
Daniela Kasulke (until 5/02)
Sebastiaan H. Meijsing (until 6/02)
Stefanie Seitz
Matthias Sieber (since 9/03)

Diploma students:
Anya Elstner (January – September 02)
Muna Krings (September 02 – February 03)
Corinna Schirling (since 4/03)
Stefanie Seitz (April – October 2000)

Technicians:
Anne Barduhn (until 6/03)
Uta Marchfelder

Summary

The expression and integrity of genetic information is directly coupled to the packaging of DNA into higher-order chromatin in the eukaryotic nucleus. Our aim is to understand the molecular basis of how chromatin controls gene expression. We have identified a novel mechanism for the reestablishment of histone acetylation patterns on chromatin after DNA replication. Furthermore, we have found a new targeting mechanism for heterochromatin, and we have discovered a regulatory function for amino-terminal protein acetylation in gene silencing. Taken together, our findings reveal a complex interplay of protein factors and their modifications in establishing functional domains within the genome.

Current state of research

The organization of DNA into chromatin and chromosomal structures plays a central role in many aspects of cell biology and development in eukaryotes. Processes ranging from chromosome stability and segregation to gene expression are intimately linked to chromatin configuration. Chromatin function is modulated by posttranslational modifications on the basic unit of chromatin, the nucleosome. Perhaps best studied is the influence of histone acetylation on transcription initiation, whereas histone methylation, phosphorylation and ubiquitination regulate various other aspects of chromatin biology. A current model posits that combinations of modifications are recognized and bound by effector proteins that translate such epigenetic patterns of nucleosome modifications into a gene expression state.

We are studying the relationship between chromatin structure and gene repression in the model organism *Saccharomyces cerevisiae*. In *S. cerevisiae*, repressed genome regions are found at the cryptic silent mating-type loci *HML* and *HMR*, in subtelomeric regions

and at the ribosomal DNA locus. One hallmark of these regions is the binding of the heterochromatic proteins Sir3 and Sir4 to unacetylated nucleosomes. Histone deacetylation in these regions is accomplished by Sir2, an NAD-dependent histone deacetylase that is essential for all forms of silencing in yeast. The focus of our research is to understand how repressive chromatin structures are targeted to a particular genomic region, how these structures are established and maintained during DNA replication, and how histone and protein modifications influence their structure and function.

Results and their significance

Reestablishment of epigenetic patterns of histone modification after DNA replication and chromatin assembly

The duplication of chromatin during the cell cycle requires that epigenetic patterns of histone modifications be reestablished on the newly formed chromatin after DNA replication. Chromatin acetylation coupled to replication is essential in order to ensure that repressor proteins like Sir3 and Sir4 don't spread inappropriately on unmodified nucleosomes in chromatin. We have gained mechanistic insight into this process by uncovering a novel interaction of the chromatin assembly factors CAF-I and Asf1 with the histone acetyltransferase complex SAS-I. We have shown that SAS-I acetylates lysine 16 of histone H4 (H4 K16) both *in vivo* and *in vitro*. Interestingly, Sir3/4 protein binding to chromatin is particularly sensitive to acetylation at this position. We propose that the SAS-I complex is recruited to the freshly assembled chromatin through CAF-I or Asf1 in order to acetylate H4 K16 in a global fashion, which prevents the spreading of heterochromatin components into euchromatic genome regions (Figure 1). Thus, this represents a new class of histone acetylation that is distinct from the known classes of acetylation like transcription initiation or elongation coupled acetylation. Furthermore, since other histone modifications like methylation or deacetylation are also reset after replication, we likewise propose that other chromatin modifying activities are recruited to new chromatin by their interaction with chromatin assembly factors.

Novel functions for the SAS-I histone acetyltransferase complex

Our work described above has firmly established a role for SAS-I in gene silencing and chromatin assembly. We have further asked whether SAS-I carries out other, hitherto unknown functions, by searching for new interaction partners of the SAS-I components. We found that SAS-I interacted with the centromeric histone H3 variant Cse4. Cse4 replaces H3 in the nucleosome at the yeast centromere and is essential for its structure and

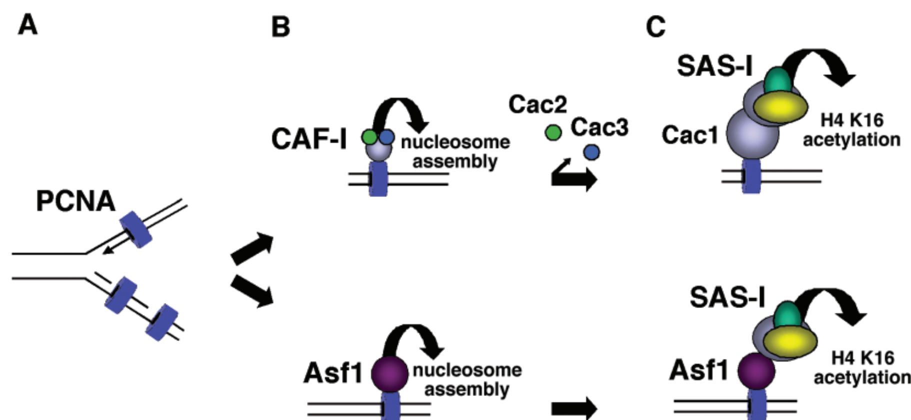


Figure 1: Model for the recruitment of the SAS-I complex to newly assembled chromatin. (A) The ring-shaped PCNA trimer associates with DNA and enhances DNA polymerase processivity during DNA replication. (B) After replication, PCNA remains topologically linked to the replicated DNA. PCNA recruits CAF-I and Asf1 to assemble nucleosomes onto the DNA. (C) Upon nucleosome assembly, Cac1 and Asf1 remain associated with chromatin and recruit the SAS-I complex to acetylate histone H4 K16.

function. In its C-terminal half, Cse4 is most similar to histone H3, but unlike conventional histones, it contains a long (127 amino acids) amino-terminal tail that interacts with kinetochore components, for instance the centromeric protein Ctf19. The observation that SAS-I interacted with Cse4 implies a function for SAS-I at the centromere. Indeed, we found that the deletion of *SAS2* (*sas2Δ*) interfered with plasmid segregation in yeast strains whose kinetochore was compromised by additional mutations. Furthermore, we found that SAS-I interacted with the amino-terminal part of Cse4, and that *sas2Δ* abrogated the interaction between Cse4 and Ctf19. One interpretation of these findings is that the SAS-I complex has a structural role at the centromere. Alternatively, since SAS-I is an enzyme, it may acetylate Cse4 and thus may modify its role at the centromere.

Furthermore, we have discovered an interaction between SAS-I and nuclear import factors. Sas5 interacted with the importin β-like factor Pse1, which serves as a shuttle for proteins synthesized in the cytoplasm to reach their nuclear destination. Sas5 lacks a classical nuclear localization signal (NLS), which fits well with the fact that importin β-like factors are specialized to transport such proteins. Significantly, Sas5 concentration in the nucleus was reduced in strains mutated in *PSE1* or *KAP123,* which encodes a second importin β-like protein, whereas other importin β mutants did not affect Sas5 localization. Interestingly, both mutants also reduced the nuclear concentration of Sas2, but not Sas4. Thus, we hypothesize that the complex components are transported individually into the nucleus, where they are then assembled into a functional complex.

Moreover, we found the SAS-I complex to interact with the protein Tgs1. Tgs1 is an RNA methyltransferase that methylates the cap structure of small nuclear and nucleolar RNAs which classically are required for splicing. This interaction suggests a functional link between the processes of histone acetylation and RNA processing, which we are currently investigating.

Regulation of ORC silencing function by amino-terminal protein acetylation

In recent years, the function of a variety of posttranslational protein modifications in chromatin has been elucidated, whereas some protein modifications have remained poorly characterized. One such modification concerns the N-terminal processing of proteins after synthesis at the ribosome. Depending on the penultimate amino acid of a protein, the initiator methionine is cleaved off, and the newly exposed residue is then acetylated by one of several N-terminal acetyltransferases. Hence, this type of acetylation differs chemically from the well characterized acetylation of lysine side chains in histone acetylation, and is also expected to be functionally distinct.

Interestingly, one of the N-terminal acetyltransferases in yeast, the Nat1/Ard1 complex, has long been known to be required for gene silencing, thus implying that one or several silencing proteins require N-terminal acetylation for full function. Genetic analyses led us to postulate that the large subunit of the Origin Recognition Complex (ORC), Orc1, is one such substrate. ORC is required to target silencing complexes to the *HM* loci and the telomeres. We demonstrated that Orc1 is fully acetylated in wild-type cells and completely unacetylated in *nat1Δ* cells. Furthermore, the mutation of the penultimate alanine of Orc1 to amino acids that abrogated its ability to be acetylated by Nat1/Ard1, also caused silencing defects. Taken together, our experiments showed that Orc1 was a target of Nat1/Ard1, and that the lack of acetylation compromised Orc1's silencing function. Hence, we have discovered a novel role for N-terminal protein acetylation in regulating the role of ORC in silencing. Since the effect of these *orc1* mutations on silencing is weaker than the effect of *nat1Δ*, there may be other silencing relevant targets for Nat1/Ard1, and we are currently evaluating additional candidates.

Characterization of a novel silencer binding factor

Repression of the mating-type genes located at *HML* and *HMR* is exerted by control elements termed silencers. In essence, they consist of combinations of binding sites for the replication initiator ORC, the telomere binding factor Rap1, and the Abf1 protein, which together recruit the heterochromatic Sir proteins to establish repressive chromatin. One silencer element, the so-called *HML*-D region within the *HML*-E si-

lencer, has so far remained uncharacterized. We hypothesize that *HML*-D contains a binding site for an as yet unknown silencer binding protein. We have narrowed down *HML*-D to a 14-basepair core sequence, and we have taken genetic and biochemical approaches in order to identify the putative D-binding protein. We have preliminary evidence that the Sum1 protein binds to *HML*-D. Sum1 is well known as a repressor protein that binds to middle sporulation element (MSE) and represses sporulation genes during vegetative growth. We found that the *SUM1* deletion caused a predicted set of silencing defects at *HML*, but not at *HMR*, which lacks the "D" element. Sum1's function in *HML* silencing has so far not been recognized. Hence, with our studies, we are uncovering a novel function for Sum1 and are expanding our knowledge of how heterochromatin components are targeted to specific regions within the genome.

Future directions

Understanding the relationship between genome sequence, epigenetic patterns of chromatin modification and gene expression will become increasingly important in the era of post-genomic science. Our goal is to obtain an integrated view of the composition and chemical modifications of chromatin in all genomic regions and to relate them to gene expression states in order to dissect the functional organization of the eukaryotic genome. In the future, we will make use of systematic approaches to investigate chromatin modifications and gene function on a genome-wide scale in *S. cerevisiae*. Since many of the components are evolutionarily conserved, it will be important to determine whether they are functionally linked in larger eukaryotes. Thus, in the long term, we will expand our studies to multicellular organisms (flies, worms) in order to investigate the influence of chromatin states on genome structure and development on an organismal level. Taken together, we will thus provide new insights into the mechanism of assembly and propagation of epigenetic information in eukaryotes, and how it controls the expression and integrity of genetic information.

General information

Publications 1998 – 2003

Margot JB, **Ehrenhofer-Murray AE,** Leonhardt H (2003). *Interactions within the mammalian DNA methyltransferase family*. BMC Molecular Biology 4(1):7

Marchfelder U, Rateitschak K, Ehrenhofer-Murray AE (2003). *SIR-dependent repression of non-telomeric genes in Saccharomyces cerevisiae?* Yeast 20(9): 797-801

Gautschi M, Just S, Mun A, Ross S, Rücknagel P, Dubaquie Y, **Ehrenhofer-Murray AE,** Rospert S (2003). *The yeast Nα-acetyltransferase NatA is quantitatively anchored to the ribosome and interacts with nascent polypeptides*. Mol Cell Biol (in press)

Kasulke D, Seitz S, Ehrenhofer-Murray AE (2002). *A role for the Saccharomyces cerevisiae RENT complex protein Net1 in HMR silencing*. Genetics 161: 1411-1423

Grünweller A, Ehrenhofer-Murray AE (2002). *A novel yeast silencer: The 2 micron origin of Saccharomyces cerevisiae has HST3-, MIG1- and SIR-dependent silencing activity*. Genetics 162: 59 - 71

Meijsing SH, Ehrenhofer-Murray AE (2001). *The silencing complex SAS-I links histone acetylation to the assembly of repressed chromatin by CAF-I and Asf1 in Saccharomyces cerevisiae*. Genes Dev 15: 3169-3182

Ehrenhofer-Murray AE, Kamakaka RT, Rine J (1999). *A role for the replication proteins PCNA, RF-C, polymerase epsilon and Cdc45 in transcriptional silencing in Saccharomyces cerevisiae.* Genetics 153: 1171-1182

Selected invited talks and seminars 1998 – 2003

Silencing speaks up: Epigenetic mechanisms of gene regulation in the yeast Saccharomyces cerevisiae, EMBL Heidelberg, 6/03

SAS and CAF: Restoration of histone modification patterns after DNA replication, Keystone Meeting "Enzymology of Chromatin and Transcription", Santa Fe, NM, 3/03

SAS and CAF: A link between chromatin assembly and histone acetylation, DFG Meeting Schwerpunkt Epigenetik, Berlin, 11/02

Resetting of epigenetic marks after replication: Interaction between SAS-I and chromatin assembly factors, Deutsche Hefetagung Ober-Ramstadt, 9/02

SAS and CAF: Connections between histone acetylation and chromatin assembly, EURESCO Conference on Gene Transcription in Yeast, Castelvecchio Pascoli, Italy, 5/02

SAS and CAF: A link between histone acetylation and chromatin assembly, FASEB Meeting on Chromatin and Transcription, Snowmass, CO, USA, 7/01

A link between histone acetylation and histone acetylation: The SAS-I complex interacts with the chromatin assembly complex CAF-I, Jacques Monod Conference on Signaling & Control of Transcription, Aussois, France, 6/01

The acetyltransferase homolog Sas2 in transcriptional silencing in Saccharomyces cerevisiae, University Center of Molecular Pathology, University of Umea, Sweden, 10/00

ORC and other replication factors in transcriptional silencing in S. cerevisiae, 23rd Annual Meeting of the German Society of Cell Biology, Rostock, Germany, 3/99

The Origin Recognition Complex, the acetyltransferase homolog Sas2 and other replication proteins in transcriptional silencing in S. cerevisiae, EMBO Workshop, Coupling of DNA Replication to Cell Growth, Geilo, Norway, 6/98

Teaching

Grundvorlesung Genetik, WS 01/02 + 02/03, 1 SWS, Humboldt University Berlin

Blockpraktikum "*Methoden der Hefegenetik*", WS 2001/02, WS 2000/01, SS 2000, 4 SWS, Humboldt University Berlin

Vorlesung (Hauptstudium) "*Molekulare und zelluläre Biologie der Hefe Saccharomyces cerevisiae*", SS 2001, 2 SWS, Humboldt University Berlin

Vorlesung (Hauptstudium) "*Epigenetische Mechanismen der Genregulation*", WS 2000/01, 1 SWS, Humboldt University Berlin

State doctorate (Habilitation)

Ann E. Ehrenhofer-Murray, *On the mechanisms of transcriptional repression in the yeast Saccharomyces cerevisiae*, Humboldt University Berlin, January 2003

Theses

Sebastiaan H. Meijsing, *The silencing complex SAS-I links histone acetylation to the assembly of repressed chromatin by CAF-I and Asf1 in Saccharomyces cerevisiae*, PhD Thesis, Humboldt University Berlin, January 2002, sponsored by the Max-Planck-Society

Daniela Kasulke, *Die Rolle der RENT-Komponente Net1 in der HMR Repression der Hefe S. cerevisiae*, PhD Thesis, Humboldt University Berlin, May 2002, sponsored by the Max-Planck-Society.

Stefanie Seitz, *Interaktion des centromerischen Histon H3-Homologs Cse4 mit der Acetyltransferase Sas2 und dem Chromatin Assembly Faktor CAF-I aus Saccharomyces cerevisiae*, Diploma Thesis, Humboldt University Berlin, October 2000

Anya Elstner, *Charakterisierung von Chromatin-Assemblierungsfaktoren in Saccharomyces cerevisiae*, Diploma Thesis, Humboldt University Berlin, September 2002

Muna Krings, *Interaktionen zwischen Chromatin-Assemblierungsfaktoren und Histondeacetylasen in S. cerevisiae*", Diploma Thesis, Humboldt University Berlin, February 2003

Appointments, scientific honors & memberships

Member of the Deutsche Gesellschaft für Genetik

Member of the Deutsche Gesellschaft für Biochemie und Molekularbiologie

Member of the Genetics Society of America

Member of the American Association for the Advancement of Science

External funding

Characterization of the silent mating-type locus HML of S. cerevisiae: Identification of a silencer binding factor and of a silencing protein that is regulated by N-terminal acetylation (DFG EH194/1-1, 1-2, Staff funded: Horst Irlbacher, Stefanie Seitz)

Molecular control of skeletal development

Head:
Dr. Andrea Vortkamp
Phone: +49 (0)30-8413 1332
Fax: +49 (0)30-8413 1130
Email: vortkamp@molgen.mpg.de

Scientists:
Eleonora Minina (until 11/2003)
Manuela Wülling (since 10/2002)

Graduate students:
Lydia Koziel
Milana Tchinenkova
Andreas Ratzka

Technicians:
Conny Kreschel
Melanie Kunath
Sabine Schneider

Students:
Theresa Bergann
Daniela Kosslick
Franziska Zabel

Summary

The aim of my research is the analysis of the signaling network controlling embryonic bone formation. Using mouse mutants and an organ culture system for embryonic limb explants we have for the first time integrated three signaling systems, the Ihh/PTHrP, BMP and FGF signaling systems, into a common control network. These investigations led to a new understanding of the molecular origins of Achondroplasia, which results from activated FGF signaling. They furthermore identified the BMP signaling pathway as a new target to treat Achondroplasia.

To understand signal propagation in the growth plate we have started to investigate the interaction of Ihh with the extracellular matrix. We found that heparan sulfates sequester Ihh signals, strongly indicating that Ihh can act as a long range signal. In addition these studies revealed activated Ihh signaling as the likely cause for the development of the human 'Hereditary Multiple Exostoses Syndrome'. We have started to extend our studies on signal interactions and signal transport to transcription factors regulating downstream gene expression.

Current state of research in the field and significance

The vertebrate skeleton is a complex organ necessary for the survival and the quality of vertebrate life. This is reflected in the large number of inherited disorders characterized by malformations of the skeleton. Moreover, age related bone diseases affecting for example bone stability (Osteoporosis) or the joint cartilage (Osteoarthrosis), have become a new focus of scientific research. The aim of my laboratory is to decipher the control mechanisms regulating embryonic bone formation. We hope that such an understanding will not only lead to new insight into developmentally derived skeletal disorders but will ultimately result in new ways to treat adult bone diseases by reactivating the embryonic program *in vivo* or in stem cell cultures.

Most of the bones of the skeleton are formed by endochondral ossification, a multistep process in which a cartilage skeleton is initially formed, that is later replaced by bone. Although endochondral ossification has been extensively studied on a morphological level, the various signaling systems regulating this complex process and their interactions are just being elucidated. We are concentrating our analysis on the early steps of endochondral ossification, when chondrocytes proliferate and differentiate into hypertrophic chondrocytes, which are subsequently replaced by bone (Figure 1). As longitudinal growth of endochondral bones is dependent on the proliferation and hypertrophic differentiation of chondrocytes, the tight regulation of these two steps is crucial to balance growth and stability of the bones.

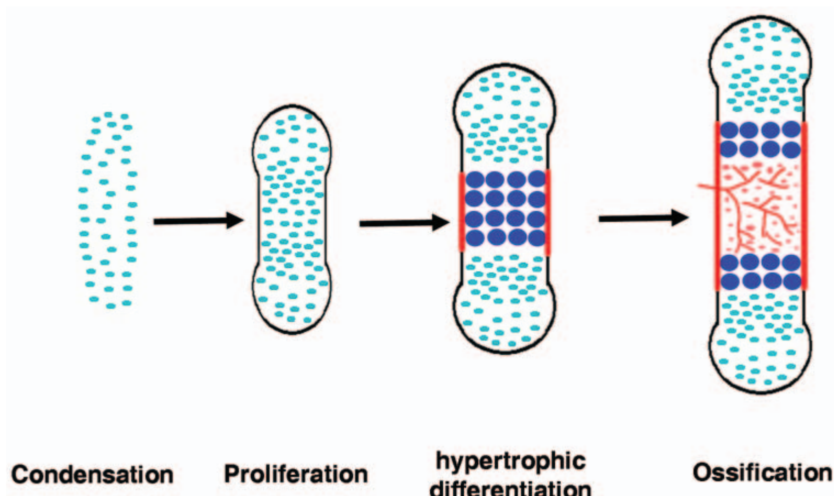


Figure 1: Endochondral Ossification - mesenchymal cells condense and differentiate into chondrocytes, which form cartilage elements, the precursors of the later bones (turquise). The cartilage elements are surrounded by a layer of fibroblastic cells, the perichondrium (black). Starting from the center of the cartilage anlagen chondrocytes differentiate into a hypertrophic chondrocytes (blue). The hypertrophic region is invaded by blood vessels (Red) osteoclast and osteoblasts, which start to replace the hypertrophic chondrocytes by bone (red) and bone marrow (red).

Results

Interaction of signaling systems controlling chondrocyte differentiation

My previous work on Ihh has uncovered a first important feedback loop in which Indian hedgehog (Ihh) expressed in the differentiating chondrocytes and Parathyroid Hormone related Protein (PTHrP) expressed in the periarticular chondrocytes interact to regulate the onset of hypertrophic differentiation. To integrate the Ihh/PTHrP signaling system with that of other signaling pathways regulating chondrocyte differentiation we have established a culture system for embryonic limb explants. This system allows the epistatic analysis of different signaling systems by co-treatment of explants with combinations of growth factors and by utilizing limbs of various mutants as source for the explants. We have for the first time integrated three signaling systems, that of Ihh/PTHrP, FGFs and

BMPs, into a common control network (Figure 2). We demonstrated that BMP and FGF signals antagonize each other in regulating at least three distinct steps of chondrocyte development. They regulate (1) chondrocyte proliferation independent of the Ihh/PTHrP system, (2) the onset of hypertrophic differentiation by acting upstream of the Ihh/PTHrP system and (3) the process of hypertrophic differentiation independent of Ihh/PTHrP (Minina et al. 2001, 2002).

These investigations led furthermore to a new interpretation of the molecular origin of achondroplasia, the most common form of human dwarfism, which results from activated FGF signaling. In contrast to the established model that activated FGF signaling in Achondroplasia delays hypertrophic differentiation we could demonstrate that it in fact accelerates this process, a finding of high importance for the development of specific treatment strategies. Building on our signaling network we could consequently demonstrate that BMP signaling rescues the reduced regions of proliferating and hypertrophic chondrocytes in a mouse model for achondroplasia implicating manipulation of the BMP signaling system as a new target to treat Achondroplasia (Minina et al., 2002).

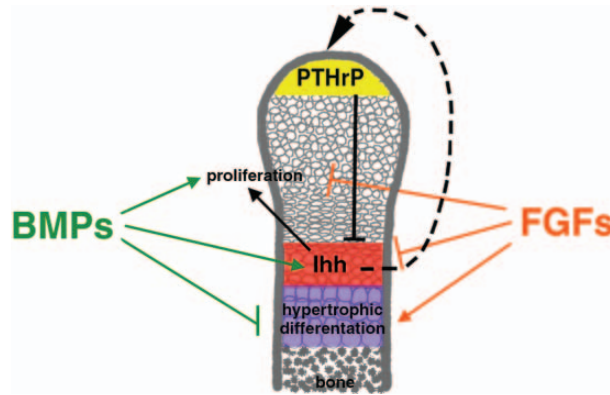


Figure 2: Ihh/PTHrP, BMP und FGF signals interact to regulate chondrocyte differentiation.

This work has demonstrated that our explant system provides a unique, powerful tool for dissecting the regulation of chondrocyte differentiation. Accordingly, we have started to extend this control network by integrating further secreted factors like TGF-ßs, Wnts and others. Preliminary results indicate that TGF-ß, like FGFs, accelerate hypertrophic differentiation. In addition we have started to explore techniques to introduce siRNA into the organ cultures, a technique, which - if successful - will enable us to rapidly analyze the function of newly identified genes before generating mouse models.

Signal propagation in the growth plate

As described above a large number of growth factors, each produced in a discrete location, interact to regulate chondrocyte proliferation and differentiation. To understand how these signals relate to one another one must have an understanding of how their respective ranges of action are determined. To this end, we have started to investi-gate the role of the extracellular matrix (ECM) in Ihh propagation. The glycosyl transferase Ext1 is one of the key enzymes for the synthesis of heparan sulfates (HS). Mutations in Ext1 in human result in benign bone tumors and short stature (Heritable multiple exostoses' (HME)). We are analyzing a gene trap mouse line carrying a hypomorphic allele of Ext1, which leads to reduced HS synthesis. These mice are characterized by delayed hypertrophic differentiation of chondrocytes. Analysis of the Ihh/PTHrP system revealed an activation of Ihh signaling. Correspondingly, treatment of limbs in culture with heparin restricts Ihh signaling in wild type and mutant animals and blocks PTHrP expression. In contrast FGF signaling seems to be not affected at the stages analyzed (Koziel, in preparation).

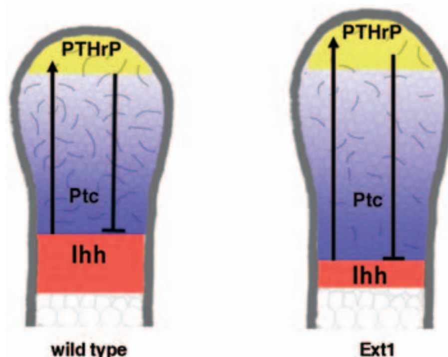


Figure 3: Heparansulfates restrict Ihh signaling.

Several important conclusions can be drawn from these experiments: 1) Although the Drosphila homolog of Ext1has been shown to be necessary for hedgehog transport Ext1 dependent HS in mice seem to restrict Ihh signaling and might thus regulate the establishment of an Ihh signaling gradient. 2) In contrast to the current model, which predicts a secondary mediator, our experiments strongly indicate that Ihh travels through the growth plate to directly induce PTHrP expression. Culture experiments, demonstrat-

ing that neither of the predicted signals, BMPs or TGF-ßs, can activate PTHrP expression in absence of Ihh signaling support this result (Minina et al., 2001; Kreschel, unpublished). 3) Activated Ihh signaling acting either on neighboring chondrocyte or on the flanking perichondium is the most likely cause for the development of exostoses in human. These investigations have for the first time addressed the role of the ECM in Ihh distribution and have resulted in a new understanding of Ihh acting as a long range signal.

We are planning to extend these studies to other growth factors that depend on interactions with HS. Because of its size the developing bone seems to be a very sensitive model to investigate long distance signaling events, and results from such investigations might be applicable to other organs.

Transcription factors acting downstream of Ihh

To understand how signals regulate gene expression it is necessary to investigate the downstream transcription factors. Zinc finger transcription factors of the Gli family, like Gli3, act downstream of hedgehog signaling. We have started to analyze the specific roles of Gli3, which can act as an activator and repressor, in regulating chondrocyte proliferation and differentiation.

Trich-rhino phalangeal-syndrome affects craniofacial and skeletal development in human. During cartilage development the underlying gene Trps1, a GATA zinc finger transcription factor is expressed partially overlapping with PTHrP and Ptc. (Kunath et al. 2002). First analyses of Trps1 null mice indicate a delay in hypertrophic differentiation. After a detailed analysis of the Trps-/- phenotype it will be highly interesting to investigate a possible interaction of the Ihh signaling system and Trps1.

Identification of new genes regulating endochondral ossification

The experiments described above are focused on the analysis of known genes. However, we expect that a large number of genes regulating bone development has not been identified yet. Using PCR based subtraction approaches we have identified more than 20 genes with specific expression patterns in the developing skeletal elements. One of the most interesting candidates is highly conserved and exclusively expressed in the developing bone. We have started to generate gain and loss of function mutants hoping that these will give new insight into how Ihh regulates the ossification process.

Another gene, PERP, which is expressed overlapping with Ihh, has originally been isolated as a target of p53. We found PERP expression overlapping with both, p53 and p63, indicating that PERP might act downstream of both genes. Accordingly we found PERP expression reduced in the skin p63-/- mice (Lintermann, in preparation).

Four-jointed (Fj) is a type II transmembrane protein, which is widely expressed in the mouse embryo including the central nervous system, joints and tendons (manuscript in preparation). We have deleted Fjx in mice but did not detect a phenotype on a 129SvEv background. We are therefore planning to reinvestigate the phenotype on a C57B6 background.

Gene expression profiling

To be able to analyze gene expression in a broader way we have started to carry out complex hybridizations on cDNA chips (Affimetrix). We plan to establish gene expression profiles of limb cultures, in which different signaling pathways have been manipulated. In addition to isolating new cartilage specific genes, we hope to identify groups of genes that react to different signals in similar ways. Recognizing such groups will extend the understanding of the signaling network and facilitate the integration of new candidates in the future.

Interaction of positional information and bone differentiation

From a developmental perspective, a key question that is still poorly addressed is how patterning of the skeleton is linked to the process of bone formation. Most differences between skeletal elements arise by differential growth after the initial cartilage anlagen are laid down. Thus the signals that regulate bone formation are likely points at which positional information might act to regulate the shape of the bones. Towards

this end I plan to analyze mouse mutants in which the skeletal anlagen form normally, but certain elements fail to develop the proper final bone structure as for example the Hox mutant ulnaless. We have started to analyze the specific steps at which bone formation is disturbed by gene expression analysis in situ and on chips. Subsequently we will try to rescue the phenotypes by manipulating the affected downstream signaling systems.

Goal

The goal of my laboratory is to identify the network of signaling systems regulating embryonic bone formation. I plan to understand the specific function of each of these signals and to place them into the context of the control network of genes regulating skeletal development. In addition to the interaction of signals we will concentrate our studies on the role of the ECM on the distribution of growth factors and on the regulation of gene expression by downstream transcription factors. We will further use gene expression analysis to identify the majority of genes regulating chondrocyte differentiation and to investigate gene regulation in a global way. In the long run we will extend these studies to mechanisms translating positional information into a bone pattern. The combination of the experimental approaches used should result in an in depth understanding of the basic mechanisms of bone formation and ultimately lead to new insight into the molecular origins of bone diseases.

General information

Publications 1998-2003

Zhou H, Weskamp G, Chesneau V, Sahin U, **Vortkamp A**, Horiuchi K, Chiusaroli R, Hahn R, Wilkes D, Fisher P, Baron R, Manova R, Basson CT, Hempstead B & Blobel CP (2003). *Essential role for ADAM19 in cardiovascular morphogenesis*. Mol Cell Biol (in press)

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Oral presentations on conferences 1998-2003

5th EMBL Mouse Molecular Genetics Meeting. Heidelberg (2003)

Gordon Conference: Cartilage Biology and Pathology. Ventura, USA (2003)

1st Wittgenstein Conference. Lucca, Italy (2002)

2nd European Conference on Bone Morphogenetic Proteins. Zagreb, Kroatia (2002)

14th International Congress of Developmental Biology. Kyoto, Japan (2001)

Basic and Applied Research in Skeletal Tissue Engineering: Perspectives. Camogli Genova, Italy (2001)

Belgische Vereniging voor Biochimie en Moleculaire Biologie. Antwerpen, Belgium (2001)

3. MSD Kolloquium "Seeoner Gespräche". Bad Sarow (2000)

Deutsche Gesellschaft für Genetik: Genetik der Entwicklung. München (1999)

Sulzer Surlej Meeting on Cartilage Biology. Surlej Silvaplana, Switzerland (1999)

4th International Skeletal Dysplasia Meeting. Baden Baden (1999)

Ernst Schering Research Foundation, Workshop 29, Of Fish, Fly Worm and Man: Lessons from Developmental Biology for Human Gene Function and Disease. Berlin (1999)

EMBO workshop on Skeletal Development. Heidelberg (1998)

Molecular Signaling in Development, Cell Differentiation and Proliferation. Tokyo, Japan (1998)

Teaching

Practical course and lecture *Biologie für Mediziner*, Humboldt University Berlin, SS 2003 (2x3SWS), SS 2001 (2x3SWS), WS 2000/01 (1x3SWS), SS 2000 (2x3SWS), WS 1999/00 (1xSWS)

State doctorate (Habilitation)

Andrea Vortkamp, *Molekulare Kontrolle der Skelettentwicklung* (submitted April 2003)

Theses

Eleonora Minina: *Interaction von Ihh/Pthlh, BMP and FGF signaling in regulating chondrocyte proliferation and differentiation*. PhD Thesis, FU Berlin (February 2002)

Markus Wenzel: *Identifizierung neuer Zielgene im Indian-Hedgehog Signalweg*, PhD Thesis, FU Berlin (March 2003)

Averhoff, P.: *Analyse der Aufgabe von EXT1 als potentieller Mediator des Hedgehog-Signales während der Chondrozytendifferenzierung im sich entwickelnden Embryo*. Diploma Thesis, Freie Universität Berlin, 2001

Appointments, scientific honors & memberships

Speaker of the Independent Junior Research Groups of the Max Planck Society (since 1999)

Member of the German Society for Developmental Biology

Member of the International Society for Developmental Biology

Organization of scientific events

Symposium der Selbständigen Nachwuchsgruppen

- October 14, 1999, Heidelberg
- October 19, 2000, Berlin
- October 18, 2001, Berlin
- October 17, 2002, Berlin

External funding

SKELNET- German Skeletal Dysplasia Network (in BMBF Rare diseases program, funding since 10/2003)

Analysis of Ext1 and its potential role in propagating Ihh signaling during endochondral ossification. (DFG Vo/620-4-1, since 2002)

'Schwerpunkt Molekulare Dysmorphogenese' Interaction of FGF and Ihh signals in regulating chondrocyte differentiation during embryonic endochondral ossification. (DFG Vo/6-2, 2000-2001)

Public relations

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