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Bone-specific master transcription factor *Runx2* regulates signaling and metabolism related programs in osteoprogenitors

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Aim. *Runx2 (AML3) transcription factor is the key regulator of osteoblastic lineage progression and is indispensable for the formation of mineral bones. Runx2 expression increases during differentiation of osteoblasts to induce osteoblast-specific genes necessary for the production and deposition of bone mineral matrix. However, Runx2 is also expressed at a lower level in early osteoprogenitors, where its function is less understood. Here we study how Runx2 determines the early stages of osteoblastic commitment using the model system of Runx2 re-introduction in mouse calvaria cells with Runx2 null background.* ***Method***. *Affymetrix analysis, Western blot analysis and quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis were employed.* ***Results***. *Gene expression profiling by Affymetrix microarrays revealed that along with the induction of extracellular matrix and bone mineral deposition related phenotypic markers, Runx2 regulates several cell programs related to signaling and metabolism in the early osteoprogenitors. Particularly, Runx2 regulates transcription of genes involved in G-protein coupled signaling network, FGF and BMP/TGF beta signaling pathways and in biogenesis and metabolism pathways of steroid hormones.* ***Conclusion***. *The data indicate that the lineage specific program, regulated by the master regulatory transcription factor, includes the regulation of cellular signaling and metabolism which may allow the committed cell to react and behave differently in the same microenvironment.*

Keywords: osteoblast progenitors, Runx2, signaling.

Introduction. Bone development, repair and remodeling homeostasis require constant differentiation of mesenchymal stem cells through osteoprogenitors to mature osteoblasts and osteocytes. Consistent with the recent discoveries that a cell fate can be programmed by only few master transcriptional regulators, the cell fate along osteoblastic lineage is regulated by Runx2 and Osterix factors, both indispensable for bone formation [1, 2].

Runx2 factor regulates expression of multiple target genes via direct activation and repression of genes transcription or by epigenetic remodeling of gene regulatory sequences [3].

Multiple *Runx2* target genes are responsible for bone mineral production at the late stages of osteoblasts maturation. However, *Runx2* expression starts very early along osteoblastic lineage, and its role at the early steps of osteogenic commitment is scarcely understood. This research is focused on the fundamental mechanisms of osteoprogenitors programming by *Runx2*. We have compared the transcriptomes of Runx2 null osteoprogenitor cells before and after their reconstitution with wild type Runx2 protein or its non-functional mutant using Affymetrix microarrays. We have

found that several clusters of functionally related genes respond to Runx2 re-introduction. They include the genes related to the osteoblast-specific signaling network, hormones biosynthesis and general metabolism. These genes define osteoblast cell identity in the bone microenvironment.

Materials and methods. *Cell culture and adenovirus infections*. Runx2 null calvaria osteoprogenitors cell line was developed previously from the fetal calvarial region of Runx2 knock-out mice by stable integration of Tert [4]. Runx2 null cells were maintained in MEM supplemented with 10 % fetal bovine serum (FBS) («Atlanta Biologicals», USA), 30 mM penicillin-streptomycin and 100 mM L-glutamine at 37 C and 5 % CO_2 humidified atmosphere. Adenoviral vectors containing cDNAs of full length Runx2 and its C-terminal deletion mutants 1–361 (C) were each transferred into the AdenoVator™ expression construct («Qbiogene», USA) from the corresponding pcDNA expression vectors described previously.

Cells were plated for infections in 6-well plates ($12.5 10^4$ cells/well). After 24 h, cells were infected with 100 MOI of each virus in 600 l of MEM media complemented with 1 % FBS for 4 h. Upon addition of 400 l media containing 1 % FBS, cells were incubated for additional 10 h.

Affymetrix analysis. Total RNA for Affymetrix analysis (and subsequent qRT-PCR validation) was isolated with Trizol reagent and purified using the RNeasy Mini Kit («Qiagen», USA). Analysis of gene expression using Mouse Genome 430 2.0 Array was performed as described earlier [5]. Data processing and sample comparisons were performed using an open source library for statistical analysis (BioConductor library for R environment; http://www.bioconductor.org). Following Robust Multi-array Average expression measurement (RMA) and background correction, the array values were subjected to quantile normalization assuming identical signal distributions in each of the arrays. Statistically significant differences between probe sets were evaluated using Student's T test ($p < < 0.05$). Functional annotation of Affymetrix probe sets and gene ontology relationships between groups of co-regulated genes were assessed using the Database for Annotation, Visualization and Integrated Discovery (DAVID 2.0) (http://david.abcc.ncifcrf.gov) [6].

Western blot analysis. Cell lysates were prepared from cell pellets that were boiled in 100 l of Direct Lysis buffer (50 mM Tris-HCl, pH 6.8, 2 % SDS, 10 % Glycerol, 12 % Urea, 25 M MG132, 100 mM DTT and 1 Complete protease inhibitors) («Roche», USA). Aliquots of each lysate (5 l) were separated in 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred using semi-wet blotting to nitrocellulose membranes («Millipore», USA). Phosphatase buffered saline (PBS) with 5 % non-fat milk was used for 1 h at room temperature to block non-specific protein binding. Primary and secondary antibodies were used at 1:2,000 dilutions for 1 h room temperature in PBS/0.1 % Tween (PBST) with 1 % milk. Signal was detected with ECL (Perkin Elmer Western Lighting Chemiluminescence Reagent Plus, «Perkin Elmer», USA). RUNX2-specific mouse monoclonal antibodies were a generous gift of Dr. Yoshiaki Ito (Institute for Molecular and Cellular Biology, Singapore). CDK2 rabbit polyclonal antibodies (SC-163) were purchased from Santa Cruz Biotechnology, Santa Cruz, USA.

Quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis. qRT-PCR was performed to validate changes for the sub-set of Runx2 responsive genes. Specific qPCR primers were designed using Primer 3 software. Total RNA for qRT-PCR assays was isolated as described, subjected to DNaseI digestion and purified using an RNA purification kit («Zymogen», USA). Aliquots of RNA (1 g) were used for reverse transcription (First strand cDNA synthesis kit, «Invitrogen», USA) with random hexamer primers. Quantitative PCR was performed with Power SYBR Green PCR Master Mix («Applied Biosystems», USA) using an automated system (Applied Biosystems 7300 Real Time PCR System) with 0.5 pmoles/ l of the specific gene primers.

Results and discussion. *Functional clustering of Runx2 responsive genes in osteoprogenitors*. To investigate *Runx2* involvement in the programming of osteoprogenitors, we used the model system of early mouse calvaria osteoprogenitors with Runx2 null background. These cells are blocked at the very early stage of osteogenic lineage because of the absence of Runx2 gene; although they deposit collagen into extracellular matrix, they do not express other phenotypic mar-

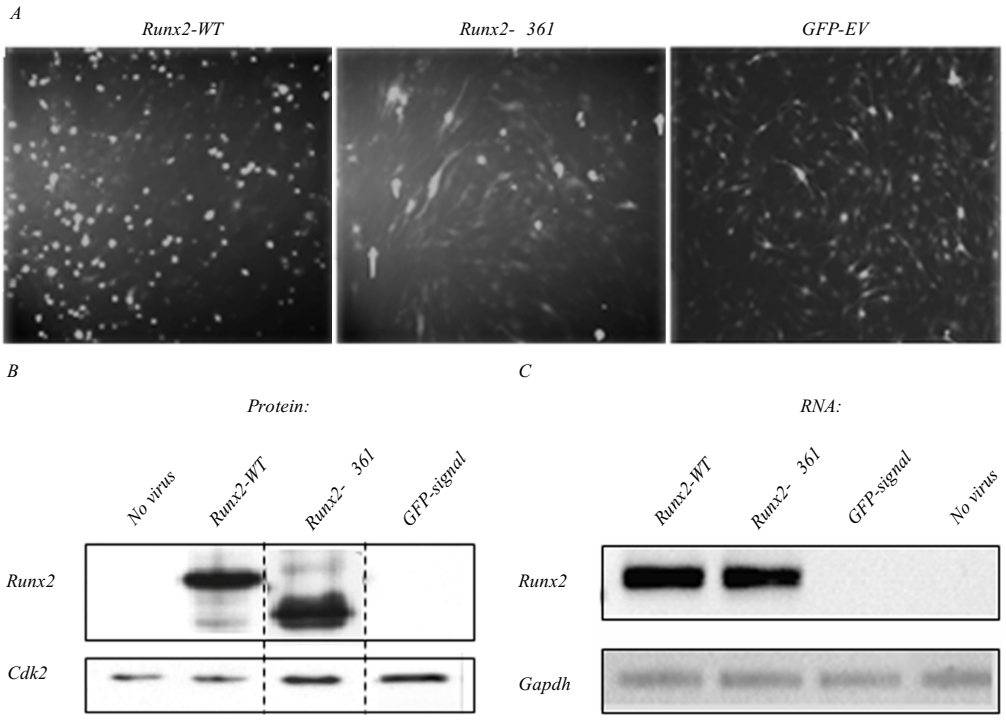

Fig. 1. *Runx2* overexpression in Runx2 null mouse calvaria cells: *A* – Runx2 wild type (WT), C-terminal truncated Runx2 mutant (aa 1–361, C) or GFP control protein were expressed via adenoviral infections in Runx2 null cells (equal infection efficiency was determined by GFP signal); *B*, *C* – exogenous Runx2 protein and RNA expression levels were detected by Western blot and RT-PCR respectively

kers and are not able to differentiate and mineralize in osteogenic media (ascorbic acid and beta-glycerophosphate). After cells reconstitution with exogenously expressed Runx2 protein the block is released and cells progress along osteoblatic lineage, differentiate to normal osteoblasts and mineralize in osteogenic media.

To assess the global changes in gene expression during the osteoblatic lineage the genome-wide Affymetrix expression profiling was performed with the investigated cells reconstituted with either wild type (WT) Runx2 protein or with its C-terminally truncated deletion mutant (Runx2- C), which is non-functional because of the absence of transactivation and repression domains (Fig. 1).

Data analysis after Runx2 WT and Runx2- C introduction revealed total 1828 probe-sets and 1438 up- and down-regulated genes (with more than 1.4 fold changes, $p < 0.05$) (Table). Functional clustering of these genes has shown that several clusters of functionally related genes are responsive to Runx2 introduction, and include the genes related to cell signaling pathways, general metabolism, transport, cell cycle and osteoblast phenotype. The changes in expression of selected subset of 180 genes were validated by quantitative PCR (qPCR) analysis and revealed a significant consistency with the data obtained by microarray.

Early osteoblast phenotypic genes are induced by Runx2 in osteoblasts progenitors. As we expected, the functional clustering of results has shown that the genes of the early/middle osteoblast differentiation encoding Osteocalcin, Osteopontin, some collagens and Matrix metalloproteinases, were robustly induced by Runx2 within 1 day after Runx2 re-introduction (Fig. 2). The genes characteristic for the late differentiation stage encoding Bone sialoprotein, Alkaline phosphatase have not revealed detectable expression (data not shown).

Matrix metalloproteinases Mmp9, Mmp13 and some other collagen matrix processing enzymes (i. e. Loxl2, Loxl3, Tissue Inhibitors of Matrix Metalloproteinases Timp1 and Timp3) were robustly induced by Runx2, while others (i. e. Timp2) were inhibited. Consistently, a sub-set of bone-related Runx2 responsive genes like secreted phosphoprotein (Spp1, Osteopontin), Bone gamma-carboxyglutamate (Bglap1, Osteocalcin) were also up-regulated. Some genes in the list encode other master fate-determining transcription factor in osteoblasts acting up- and downstream of Runx2 protein (e. g. Core binding factor beta (Cbfb), Sp7 or Osterix).

Runx2 programs bone-related signaling network in osteoprogenitor cell. Along with the cluster of osteo-

The number of genes up- and down-regulated by Runx2 in osteoprogenitors

Functional cluster	Probe-sets	Genes		
		Total	Up-regulation	Down-regulation
Total change	1828	1438	660	778
Not annotated yet	211	198	75	123
Genes with unknown function	234	191	94	97
Miscellaneous	147	121	47	74
Signaling network	268	192	75	117
Biosynthesis/metabolism	135	102	60	42
Cytokines/Inflammatory response	121	87	38	49
Growth/Cell cycle	93	70	41	29
Cytoskeleton/transport/proteins sorting	90	68	27	41
Proteasome degradation	70	57	21	36
Channels/transporters	77	56	22	34
Bone phenotypic genes	61	45	22	23
RNA binding/splicing/metabolism	51	41	27	14
Myogenic phenotype	46	36	21	15
Adhesion/Migration/communications	42	32	3	29
Steroids biosynthesis/metabolism	37	31	16	15
Heat shock/Chaperones	47	30	25	5
Apoptosis	38	30	16	14
Function in nervous system	32	26	8	18
Translation	28	25	22	3

blast phenotypic gene markers, several other big clusters of interrelated genes were responsive to Runx2. The biggest of these clusters includes genes coding for cell signaling pathways, namely transmembrane receptors, extra- and intracellular modulators of their activity, cytoplasmic secondary messengers and the nuclear effectors. Among the most affected signaling pathways we identified all known critical signaling components in osteogenesis, including FGF, BMP/TGFbeta, Wnt and small G-proteins coupled signaling (e. g. PTH/PTHrP, non-genomic steroid receptor Gpr30, cAMP signaling, growth factors FGF and EGF signaling).

We further arranged a sub-cluster of Runx2 responsive genes related to G-protein coupled signaling in osteoblasts (Fig. 3). A bunch of G-protein coupled receptors and their interacting G-proteins are up- and down-regulated in osteoprogenitors. Some of these genes are known to play a role in osteoblastic cells (e. g. non-genomic Estrogen receptor Gpr30, Gper), while the function of other G-proteins coupled receptors (e. g. Gpr54, Gpr23) in bone has to be determined. The four of the Small Regulators of G-proteins (Rgss) are repressed by Runx2: Rgs2, 4, 5 and 16 (Fig. 3). Notably, Rgs2 and Rgs4 are known as molecular switches directing the signal along different ways from PTH/PTHrP receptor [7, 8], the critical modulator of osteoblastic differentiation, growth and function [9]. Further investigation is needed to determine if these changes affect signaling pathway from PTH/PTHrP receptor during differentiation.

The second sub-cluster includes the extracellular matrix/proteoglycan genes known as the regulators of

FGF, Wnt and BMP signaling, critical in the bone cells [10, 11]. Heparane sulfate proteoglycanes (Syndecanes) physically interact with FGF receptors as well as FGF ligands and regulate signaling activity depending on the types of Heparane sulfate chains present [12]. The enzymes modifying chemical composition of Heparane sulfate chains components along with Proteoglycans co-repressors themselves are Runx2 responsive (Fig. 4), indicating that Runx2 plays a role as a modulator of the whole FGF signaling system.

Runx2 modulates steroid hormones biosynthesis and cholesterol metabolism machinery in osteoprogenitors. The second big cluster of genes responsive to Runx2 includes the genes responsible for cellular metabolism (Table), particularly for lipid/fatty acid, sugars and energy metabolism, red-ox homeostasis and cholesterol/steroid hormones biogenesis (Fig. 5). There are genes coding for the enzymes for several consecutive steps of cholesterol biosynthesis (Fdps, Lss, Cyp51, Dhcr) following by Cholesterol downstream conversion to Pregnenolone (Cyp11a1, Fdxr) are induced by Runx2. Interestingly, the genes related to bone-catabolic glucocorticoids signaling, including Glucocorticoid receptors (Nr3c1, Nr2c2) and Glucocorticoid-induced gene (Gig1, Zfp704) are inhibited by Runx2 expression (Fig. 5). Taken together these data suggest that Runx2 may promote the endogenous biogenesis of some anabolic steroid hormones derivatives of cholesterol in differentiating osteoblasts, while repressing signaling from catabolic glucocorticoids.

We performed genome-wide screening of the genes that are directly or indirectly regulated by the bone master regulatory transcription factor Runx2 at the early stage of osteoblastic commitment. We found that several interrelated functional gene programs are architected by the presence of Runx2 in osteoprogenitors, including early steps of bone matrix production, regulation of osteoblastic cell cycle, general metabolism, steroids biogenesis and the membrane/intracellular signaling network.

Genetic alterations of osteoblast specific signaling pathways that we have identified – FGF, PTH/PTHrP, BMP, TGF and Wnt signaling, cause hereditary syndromes with prominent bone abnormalities. Also, there are tremendous amount of accumulated evidences that each of above-mentioned signaling pathways plays a critical role in osteoblasts development and bone formation, both *in vivo* and *in vitro* [13].

Moreover, proper hormone signaling involving all classes of steroids is critical for bone development and homeostasis. For example deficiency of Estrogen or Androgen receptor leads to severe osteoporosis; deficiency of Vitamin D is well known to cause Rickets, while glucocorticoid treatment is associated with glucocorticoid-induced osteoporosis.

Taken together, these results guided us to a novel fundamental concept that the master transcriptional regulator (i. e. Runx2) changes representation of the total signaling network of the cell, and that such change may be a principal part of the fate-determining program to define cell identity in the environment. In other words, due to reprogramming its signaling network, Runx2 expressing osteoprogenitor reacts and behaves differently from its mother Mesenchymal stem cell in the same bone microenvironment, retaining the osteoprogenitor identity memory. The retention of such memory can be realized by chromatin remodeling (as Runx2 is a part of chromatin modification complexes) as well as by the retention of Runx2 on mitotic chromosomes through progenitor cells generations.

At the same time, using bone as a model system, it is a part of broader biological problem of understanding the complete mechanism of cell specialization and (re)programming by master regulatory factors. Still far from understanding, this question is of the critical importance in the new and fast growing field of stem cell-based regenerative medicine, which has tremendous potential for improving both healthcare and human wellbeing.

Н. М. Теплюк, В. І. Теплюк

Транскрипційний фактор *Runx2* регулює генетичні програми, пов'язані із сигналінгом і метаболізмом попередників остеобластів

Резюме

Мета. *Транскрипційний фактор Runx2 (AML3) є важливим регулятором диференціації остеобластів, необхідним для формування кісток. Експресія гена Runx2 зростає в процесі диференціації остеобластів і призводить до активації остеобласт-специфічних генів, відповідальних за продукування мінерального матриксу. Мета роботи полягала у визначенні функції гена Runx2 у попередниках остеобластів, де він експресується на досить низькому рівні.* ***Методи***. *Ми дослідили функцію гена Runx2 на ранніх стадіях розвитку остеобластів*

за допомогою модельної системи Runx2-нокаутних клітин скальпів мишей, яким вводили ген Runx2. ***Результати****. При дослідженні експресії геному за допомогою Affymetrix чипів виявлено, що разом із індукцією фенотипових маркерів відкладання зовнішньоклітиного мінерального матриксу ген Runx2 регулює декілька генетичних програм, пов'язаних із сигналінгом і метаболізмом попередників остеобластів. Зокрема, Runx2 регулює гени сигнальної мережі, зчепленої з G-білками, сигнальні шляхи FGF, BMP/TGF, а також ферментативні системи біосинтезу і метаболізму стероїдних гормонів.* ***Висновки****. Отримані дані вказують на те, що частина програми спеціалізації, яку виконує ключовий транскрипційний фактор, складається з програмування сигнальних шляхів та метаболізму клітини, дозволяючи ранньоспеціалізованій клітині реагувати та функціонувати певним чином у мікросередовищі.*

Ключові слова: попередники остеобластів, Runx2, сигналінг.

Н. М. Теплюк, В. И. Теплюк

Транскрипционный фактор *Runx2* регулирует генетические программы, связанные с сигналингом и метаболизмом предшественников остеобластов

Резюме

Цель*. Транскрипционный фактор Runx2 (AML3) является ключевым регулятором дифференциации остеобластов, необходимым для формирования костей. Экспресия гена Runx2 повышается в процессе дифференциации остеобластов, где он активирует остеобласт-специфические гены, необходимые для продукции минерального матрикса. Цель работы состояла в определении функции гена Runx2 в ранних предшественниках остеобластов, где он экспрессируется на достаточно низком уровне.* ***Методы****. Мы исследовали, как Runx2 функционирует на ранних стадиях специализации остеобластов с помощью модельной системы внедрения Runx2 в Runx2-нокаутные клетки скальпа мыши.* ***Результаты****. При изучении экспресии генома с помощью Affymetrix чипов обнаружено, что вместе с индукцией фенотипичных маркеров, отвечающих за продукцию внеклеточного матрикса, Runx2 регулирует несколько генетических программ, связанных с сигналингом и метаболизмом остеобластов. В частности, Runx2 регулирует гены сигнальной сети, сцепленной с G-белками, сигнальных путей FGF, BMP/TGF, а также системы биосинтеза и метаболизма стероидных гормонов.* ***Выводы****. Полученные данные указывают на то, что программа специализации, выполняемая ключевым транскрипционным фактором, включает программирование сигнальных путей и метаболизма клетки, позволяя раннедифференцированной клетке вести себя определенным образом в идентичной микросреде.*

Ключевые слова: предшественники остеобластов, Runx2, сигналинг.

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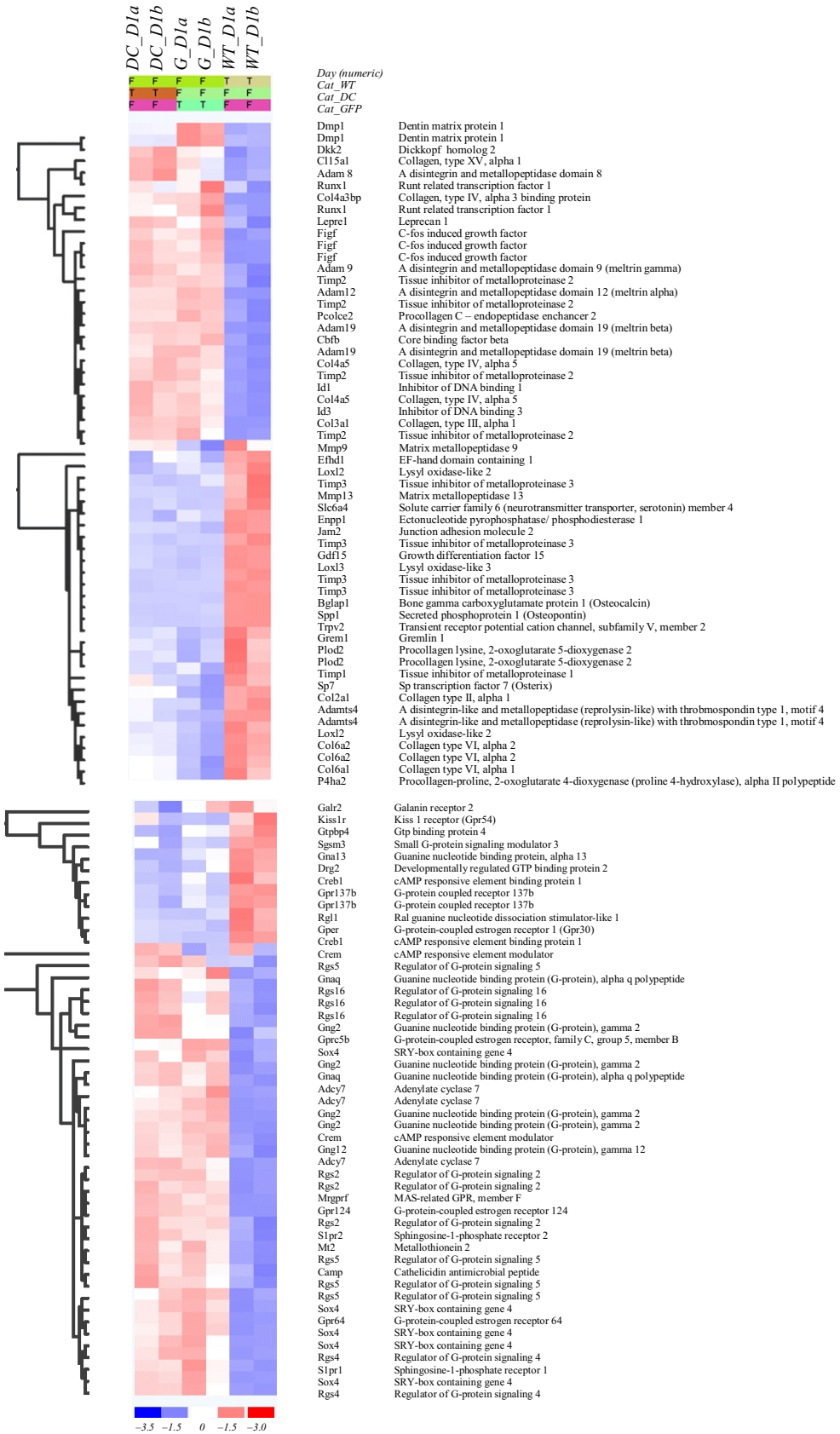

Fig. 2. Osteoblast phenotypic markers responsive to Runx2 WT versus Runx2 C non-functional mutant as was detected by functional clustering of Affymetrix microarrays data

Fig. 3. *Runx2*-responsive genes related to G-protein coupled signaling in osteoprogenitors

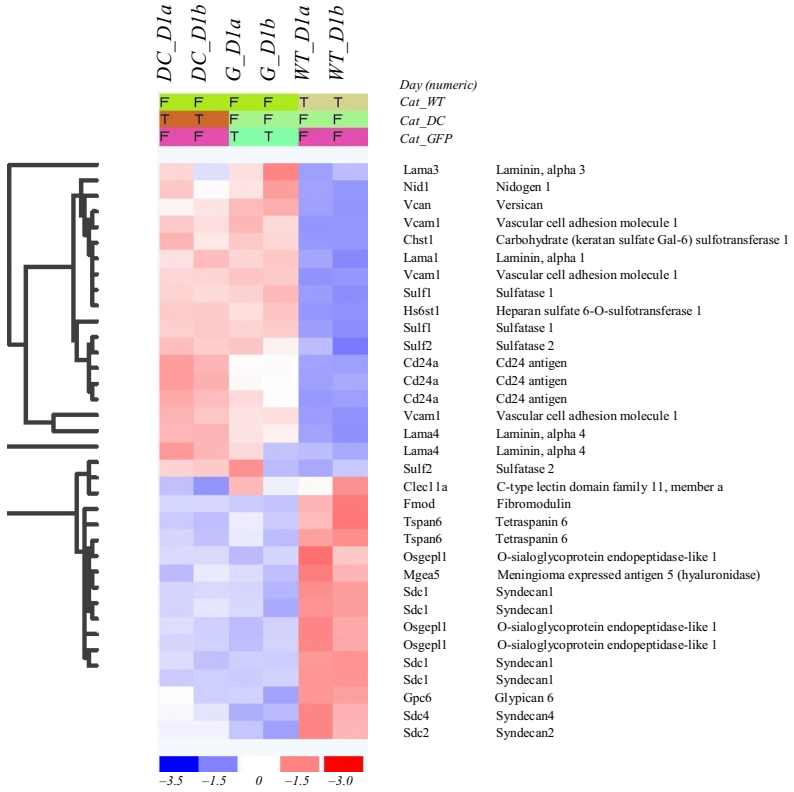

Fig. 4. *Runx2*-responsive genes related to FGF/proteoglycan signaling in osteoprogenitors

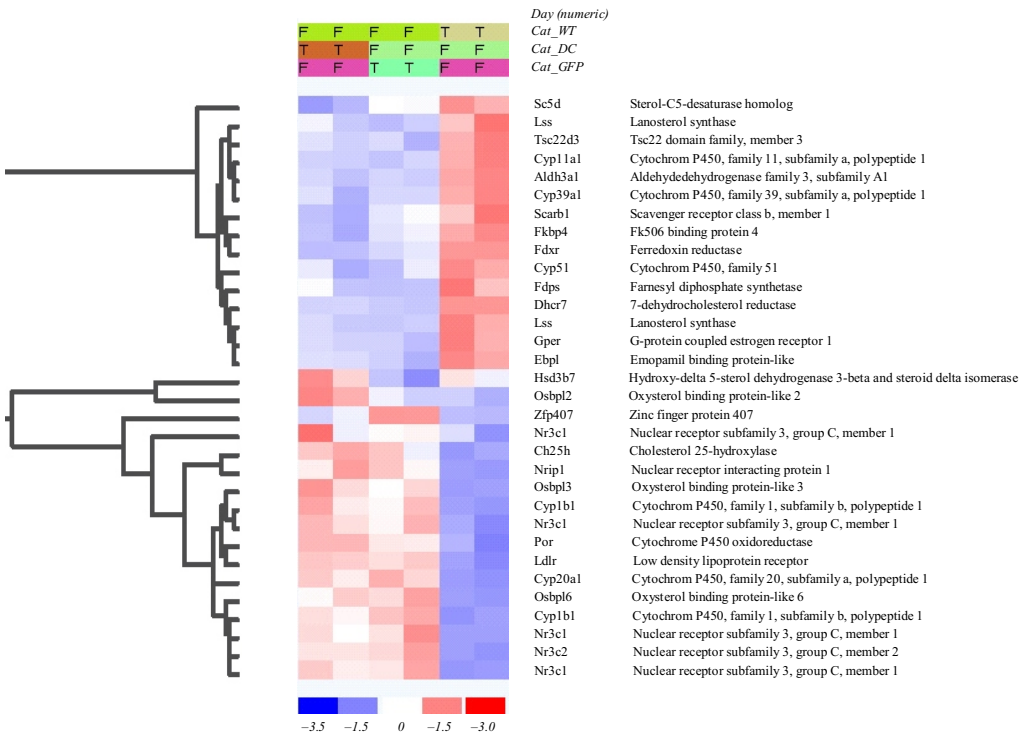

Fig. 5. *Runx2*-responsive genes related to steroid hormones signaling and metabolism in osteoprogenitors