Genes and Immunity (2012) **13**, 543–548
© 2012 Macmillan Publishers Limited All rights reserved 1466-4879/12
www.nature.com/gene

**ORIGINAL ARTICLE**

# HLA and SNP haplotype mapping in the Japanese population

H Kitajima, M Sonoda and K Yamamoto

The genes that encode the human leukocyte antigen (HLA) class I and II molecules are highly polymorphic and located in the major histocompatibility complex (MHC) region, where there is a high density of immune-related genes. Numerous studies have identified disease susceptibility in this region; however, interpretation of the results is complicated because of the strong linkage disequilibrium (LD) among HLA alleles and single-nucleotide polymorphisms (SNPs). In this study, we evaluated the correlation between the HLA alleles of 6 loci (*HLA-A, C, B, DRB1, DQB1* and *DPB1*) and 6502 SNPs within 8 Mb of the extended MHC region using 92 Japanese subjects to identify SNP single loci or haplotypes that tag HLA alleles. We found a total of 39 HLA alleles that showed strong LD ($r^2 \geqslant 0.8$) with SNPs, including 11 non-synonymous SNPs in non-HLA genes. In addition, we identified several SNP haplotypes in strong LD ($r^2 \geqslant 0.8$) with eight HLA alleles, which do not possess tag SNPs. Our detailed list of tag SNPs and haplotypes could be utilized for a better understanding of the results obtained by association studies in the Japanese population and for the characterization of the differences in LD structures between races.

*Genes and Immunity* (2012) **13**, 543–548; doi:10.1038/gene.2012.35; published online 23 August 2012

**Keywords:** HLA; tag SNP; tag SNP haplotype; disease susceptibility; linkage disequilibrium

## INTRODUCTION

The human leukocyte antigen (HLA) class I and II molecules have central roles in the differentiation of T cells in the thymus and in immune responses to foreign antigens in the peripheral lymphoid organs. The genes that encode these molecules are highly polymorphic and are located in the major histocompatibility complex (MHC) region on the short arm of chromosome 6, where immune-related genes are found at a high density with strong linkage disequilibria (LD). Numerous studies have demonstrated associations between HLA alleles and disease susceptibility in various populations.[1] In addition, recent genome-wide association studies (GWASs) have identified single-nucleotide polymorphisms (SNPs) associated with multifactorial diseases in the MHC region (GWAS catalog in the Table Browser in the UCSC Genome Bioinformatics database, http://genome.ucsc.edu/). However, the analyses were not always able to determine the primary susceptibility gene because of the strong LD among HLA alleles and SNPs. Therefore, efforts to clarify the details of the LD structure constructed with HLA alleles and SNPs residing in non-HLA genes would be required for better interpretations of the results obtained by the association studies that identify susceptibility loci in the MHC region.

Using 361 multiethnic samples including African (YRI), European (CEU), Chinese (CHB) and Japanese (JPT) samples, de Bakker *et al.*[2] produced a high-resolution HLA and SNP haplotype map in the extended human MHC region (26–34 Mb of chromosome 6). The study elucidated detailed LD structures in this region and provided information about the correlation between HLA alleles and SNPs. However, in this study, the *DPB1* locus, which is also reported to be associated with several immune-related diseases,[3–9] was not genotyped. Therefore, additional studies including *DPB1* would provide more information in this research area.

In this study, we investigated the correlation between HLA alleles of 6 loci (*A, C, B, DRB1, DQB1* and *DPB1*) and 6502 SNPs within the extended MHC region using 92 Japanese samples. The results, in combination with those from de Bakker *et al.*,[2] could be utilized for association studies in the Japanese population and for the characterization of the differences in LD structures between races.

## RESULTS

### Identification of pairs of HLA alleles and SNPs in absolute LD

The calculation of the pairwise linkage disequilibrium (LD) revealed diversity in the extent of LD between HLA alleles and SNPs, even within the same HLA locus (Figure 1 and Supplementary Figure 1). For example, the SNPs showing moderate-to-strong LD with A*33:03 were more broadly distributed than SNPs with A*24:02 (blue and red dots in Figure 1). The HLA alleles C*14:03, B*44:03, DRB1*13:02 and DQB1*06:04 that compose a common HLA haplotype together with A*33:03 in the Japanese population also showed a broad distribution of LD SNPs (Supplementary Figure 1), supporting the inheritance of the haplotype together with the surrounding SNP alleles. A total of 29 HLA alleles showed absolute LD ($r^2 = 1$) with at least one SNP (Supplementary Table 1). In the data set used in this study, there were seven types of missense SNPs among SNPs showing absolute LD with HLA alleles, which should be noted in any association studies because these SNPs might directly affect the function of the corresponding genes (Table 1). The combinations of HLA alleles and SNPs were the following: C*04:01 and three missense SNPs (rs2233976: Gly>Arg in *C6orf15*, rs130072: Arg>Gln in *CCHCR1* and rs2073724: Pro>Leu in *TCF19*); C*12:02 and one missense SNP (rs2270191: Val>Met in *C6orf15*); C*14:03 and three missense SNPs (rs2255221: Trp>Cys in *HCP5*, rs11538264: Val>Met in *PRRC2A* and rs11758242: Ser>Tyr in *LY6G5B*); B*44:03 and three missense SNPs (rs2255221, rs11538264 and rs11758242: see above); and B*52:01 and one missense SNP

Division of Genome Analysis, Research Center for Genetic Information, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan. Correspondence: Dr K Yamamoto, Division of Genome Analysis, Research Center for Genetic Information, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
E-mail: kyama@bioreg.kyushu-u.ac.jp
Received 23 April 2012; revised 17 July 2012; accepted 27 July 2012; published online 23 August 2012

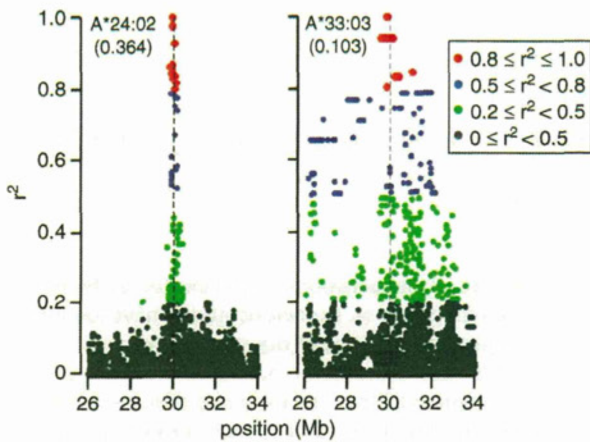



**Figure 1.** LD between SNPs and HLA-A*24:02 or HLA-A*33:03 in the extended HLA region. The SNPs across the 8 Mb extended HLA region (from 26 to 34 Mb of chromosome 6) showing weak ($0.2 \leqslant r^2 < 0.5$; light green), moderate ($0.5 \leqslant r^2 < 0.8$; blue) and strong ($0.8 \leqslant r^2 \leqslant 1.0$; red) LD were plotted. The allele frequencies of each HLA allele observed in this study are in parentheses. The dashed lines indicate the position of *HLA-A*.

**Table 1.** List of pairs between HLA allele and non-synonymous SNP in absolute LD ($r^2 = 1$)

| *HLA allele* | *SNP* | *Amino-acid substitution* | *Genes* |
|---|---|---|---|
| C*04:01 | rs2233976 | Gly > Arg | *C6orf15* |
| | rs130072 | Arg > Gln | *CCHCR1* |
| | rs2073724 | Pro > Leu | *TCF19* |
| C*12:02 | rs2270191 | Val > Met | *C6orf15* |
| C*14:03 | rs2255221 | Trp > Cys | *HCP5* |
| | rs11538264 | Val > Met | *PRRC2A* |
| | rs11758242 | Ser > Tyr | *LY6G5B* |
| B*44:03 | rs2255221 | Trp > Cys | *HCP5* |
| | rs11538264 | Val > Met | *PRRC2A* |
| | rs11758242 | Ser > Tyr | *LY6G5B* |
| B*52:01 | rs2270191 | Val > Met | *C6orf15* |

Abbreviations: HLA, human leukocyte antigen; LD, linkage disequilibrium; SNP, single-nucleotide polymorphism.

(rs2270191: see above). Besides the 29 HLA alleles, there were 10 HLA alleles that showed strong LD with SNPs ($0.80 \leqslant r^2 < 1.0$) (Supplementary Table 2). Among them, A*31:01, C*04:01, C*12:02, B*52:01 and DPB1*05:01 showed LD with missense SNP rs1116221: Glu > Lys in *TRIM31*, rs9263870: Asn > Asp in *HCG27*, rs130075: Arg > Gln in *CCHCR1*, rs130075: see above and rs11551421: Val > Met in *HLA-DPB1*, respectively. We also observed various degrees of pairwise LD between HLA alleles in the Japanese population, which is consistent with previous reports.[10] In this study, four absolute LD pairs of HLA alleles were detected: C*12:02 and B*52:01; C*14:03 and B*44:03; B*13:01 and DRB1*12:02; and DRB1*15:01 and DQB1*06:02 (Supplementary Table 3).

## LD between HLA alleles and SNP haplotypes

Among the 53 HLA alleles that did not show absolute LD with any SNPs, 3 HLA alleles exhibited absolute LD with a total of 7 haplotypes (Table 2). Among the three HLA alleles, C*14:02 and B*46:01 do not possess SNPs in strong LD ($0.80 \leqslant r^2 < 1.0$). One missense SNP (rs1116221: Glu > Lys in *TRIM31*) was linked with A*31:01 in the haplotype. Thirteen HLA alleles exhibited strong LD

**Table 2.** List of pairs of HLA alleles and SNP haplotypes in absolute LD ($r^2 = 1$)

| *HLA allele* | *Haplotype* | *SNPs in haplotype* | *$r^2$ of each SNP with the HLA allele* |
|---|---|---|---|
| A*31:01 | rs2524035(T)- | rs2524035 | 0.38 |
| | rs2844796(T)- | rs2844796 | 0.81 |
| | rs2517592(A) | rs2517592 | 0.25 |
| A*31:01 | rs9258883(C)- | rs9258883 | 0.31 |
| | rs17180570(C)- | rs17180570 | 0.35 |
| | rs2844792(A) | rs2844792 | 0.72 |
| A*31:01 | rs2524005(T)- | rs2524005 | 0.35 |
| | rs2517597(A) | rs2517597 | 0.72 |
| A*31:01 | rs12665039(C)- | rs12665039 | 0.38 |
| | rs2245420(G)- | rs2245420 | 0.72 |
| | rs12176323(T) | rs12176323 | 0.81 |
| A*31:01 | rs1116221(T)- | rs1116221 | 0.81 |
| | rs2523979(T) | rs2523979 | 0.72 |
| C*14:02 | rs4947296(C)- | rs4947296 | 0.38 |
| | rs2442736(G) | rs2442736 | 0.74 |
| B*46:01 | rs3828913(A)- | rs3828913 | 0.35 |
| | rs9296003(T)- | rs9296003 | 0.48 |
| | rs9348878(C) | rs9348878 | 0.21 |

Abbreviations: HLA, human leukocyte antigen; LD, linkage disequilibrium; SNP, single-nucleotide polymorphism.

**Table 3.** List of HLA alleles showing strong LD ($0.80 \leqslant r^2 < 1.0$) with SNP haplotypes

| *HLA allele* | *SNP* | | *Haplotype* | |
|---|---|---|---|---|
| | *Number*[a] | *Maximum $r^2$* | *Number*[b] | *Maximum $r^2$* |
| **A*02:01** | 0 | — | 2 | 0.94 |
| C*08:01 | 20 | 0.87 | 2 | 0.86 |
| C*15:02 | 3 | 0.85 | 3 | 0.92 |
| **DRB1*04:06** | 0 | — | 1 | 0.83 |
| DRB1*08:03 | 1 | 0.92 | 2 | 0.92 |
| **DRB1*14:06** | 0 | — | 1 | 0.86 |
| **DQB1*03:01** | 0 | — | 2 | 0.86 |
| **DPB1*02:01** | 0 | — | 6 | 0.90 |
| **DPB1*03:01** | 0 | — | 1 | 0.82 |
| DPB1*09:01 | 1 | 0.83 | 5 | 0.94 |

Abbreviations: HLA, human leukocyte antigen; LD, linkage disequilibrium; SNP, single-nucleotide polymorphism. The HLA alleles that possess tag haplotype but not tag SNP are shown in bold face. Note that HLA-C*14:02 and -B*46:01 that possess tag haplotype ($r^2 = 1.0$) but not tag SNP are shown in Table 2. [a]Number of single SNP loci showing strong LD ($0.80 \leqslant r^2 < 1.0$) with the HLA allele. [b]Number of SNP haplotypes showing strong LD ($0.80 \leqslant r^2 < 1.0$) with the HLA allele.

with a total of 45 haplotypes ($0.80 \leqslant r^2 < 1.0$) (Supplementary Table 4). One missense SNP (rs2523989: Val > Ile in *TRIM31*) was linked with A*31:01 in the haplotype, and two missense SNPs (rs2071554: Arg > Gln in *HLA-DOB* and rs2855430: Pro > Leu in *COL11A2*) were linked with DPB1*09:01 in the haplotype. The analyses newly identified tag haplotypes for six HLA alleles (A*02:01, DRB1*04:06, DRB1*14:06, DQB1*03:01, DPB1*02:01 and DPB1*03:01), which possess no SNPs in strong LD ($0.80 \leqslant r^2 < 1.0$) (Table 3). Thus, we identified SNP haplotypes in strong LD with eight HLA alleles, which do not possess tag SNPs. The tag haplotypes for C*15:02 and DPB1*09:01 showed stronger LD than the tag SNPs (Table 3).


© 2012 Macmillan Publishers Limited

## Tag SNPs and disease associations

The results of this study reveal that some disease-related HLA alleles possess tag SNPs in the Japanese population (Table 4). It is noteworthy that B*52:01, which denotes susceptibility to ulcerative colitis[15] and aortitis syndrome[16], possesses several tag SNPs located in the *C6orf15*, *PSORS1C1*, *CDSN*, *PSORS1C2* and *CCHCR1* region, suggesting that these non-HLA genes could be considered as candidate genes that are responsible for these diseases.

From the viewpoint of SNPs identified by GWAS in Asian populations, several tag SNPs of HLA alleles have been reported to be associated with multifactorial diseases. For Japanese subjects, the tag SNP rs9263739 of C*12:02 and B*52:01, which is located in the intron region of *CCHCR1*, was reported to be significantly associated with ulcerative colitis.[11] The tag SNP rs11752643 of DQB1*06:04 and DRB1*13:02 was reported to indicate susceptibility to coronary heart disease.[12] The association of the SNP rs987870, which composes the tag haplotype for DPB1*09:01, with pediatric asthma was also reported.[13] For Chinese Han subjects, the SNP rs2281388 showing absolute LD with DPB1*05:01 and the SNP rs4947296 composing the tag haplotype for C*14:02 were reported to be significantly associated with Graves disease[14] (Tables 5 and 6). These results suggest that the HLA alleles should be considered as indicators of susceptibility to these diseases, even in cases of non-immune-related diseases such as coronary artery disease. Thus, information about SNPs that tag HLA alleles broadens the spectrum of candidate genes in GWASs that detect associated SNPs in the HLA regions.

## DISCUSSION

This study detected tag SNPs and haplotypes for some disease-related HLA alleles in the Japanese population. Based on this data, additional candidate genes were discovered to provide a better understanding of the pathogenesis of HLA-associated diseases. Although previous studies reported that HLA-B*52:01 is associated with ulcerative colitis[15] and aortitis syndrome[16] in the Japanese population, this study detected strong LD between this HLA allele and the SNPs that are located in the major psoriasis-susceptibility locus (*PSORS1C1*, *CDSN* and *CCHCR1*). The Japanese GWAS reported that rs9263739 in *CCHCR1*, which was found to be in strong LD ($r^2=0.94$) with HLA-B*52:01 (Supplementary Table 2), was significantly associated with ulcerative colitis.[11] Elomaa *et al.*[17] reported that *CCHCR1* transgenic mice appeared phenotypically normal and that their skin was histologically indistinguishable from wild-type mice. However, the expression of genes involved in the pathogenesis of psoriasis was changed in the mice. In addition, an epidemiological study reported that psoriasis was associated with ulcerative colitis.[18] Considering that the inflammation of the mucosa and occasionally the submucosa of the colon causes ulcerative colitis, it was necessary to investigate not only HLA-B*52:01 but also the genes in the psoriasis-susceptibility locus to elucidate the pathogenesis of ulcerative colitis.

It was also important to consider the influence of HLA alleles when investigating the genes located in the extended MHC region. This study revealed that the SNP rs11538264: Val>Met,

**Table 4.** List of HLA alleles, HLA-associated diseases and the tag SNPs in the Japanese population

| *HLA risk allele (associated disease, reference number)* | *Tag SNP* | $r^2$ | *Function of SNP* | *Gene* |
|---|---|---|---|---|
| HLA-A*02:06 | rs2517830 | 1.0 | Unknown | — |
| (Graves disease[28]) | rs7760545 | 0.95 | Unknown | — |
| | rs6457109 | 0.85 | Unknown | — |
| HLA-B*51:01 (Behçet's disease[29]) | rs2442736 | 0.91 | Unknown | — |
| HLA-B*52:01 | rs2270191 | 1.0 | Missense | *C6orf15* |
| (Ulcerative colitis[15]) | rs3132550 | 0.94 | Intron | *CDSN, PSORS1C1* |
| (Aortitis syndrome[16]) | rs4410768 | 1.0 | Intron | *PSORS1C1* |
| | rs7757012 | 0.94 | Near gene 5 | *PSORS1C2* |
| | rs12364 | 0.94 | Coding synon | *CCHCR1* |
| | rs9263739 | 0.94 | Intron | *CCHCR1* |
| | rs9263749 | 0.94 | Intron | *CCHCR1* |
| | rs130075 | 0.94 | Missense | *CCHCR1* |
| | rs35718543 | 0.94 | Unknown | — |
| | rs28360997 | 0.94 | Unknown | — |
| | rs28367729 | 0.84 | Unknown | — |
| | rs2246010 | 0.84 | Unknown | — |
| | rs2844586 | 0.84 | Unknown | — |
| | rs28399987 | 0.83 | Near gene 3, near gene 5 | *MSH5, SAPCD1* |
| HLA-DRB1*09:01 (Myasthenia gravis[30]) | rs16870207 | 0.88 | Near gene 5 | *HLA-DRB5* |
| HLA-DPB1*05:01 | rs9378177 | 1.0 | Intron | *HLA-DPB1* |
| (Multiple sclerosis opticospinal form[7]) | rs11551421 | 0.96 | Missense | *HLA-DPB1* |
| (Graves disease[3]) | rs2068204 | 0.98 | Unknown | — |
| (Japanese cedar pollinosis[6]) | rs10484569 | 0.98 | Unknown | — |
| | rs2281388 | 1.0 | Unknown | — |
| | rs9296081 | 0.98 | Unknown | — |
| | rs6457713 | 0.98 | Unknown | — |
| | rs9380342 | 1.0 | Unknown | — |
| | rs9380343 | 0.98 | Unknown | — |
| | rs12174662 | 1.0 | Unknown | — |
| | rs6937034 | 1.0 | Unknown | — |
| | rs9348906 | 0.98 | Untranslated 5 | *HLA-DPB2* |
| | rs9366814 | 0.91 | Intron | *HLA-DPB2* |
| | rs2235499 | 0.82 | Unknown | — |

Abbreviations: HLA, human leukocyte antigen; SNP, single-nucleotide polymorphism. HLA alleles that have been reported to be associated with diseases but do not possess SNPs in strong LD are not shown.

© 2012 Macmillan Publishers Limited

**Table 5.** List of HLA alleles and their tag SNPs that are described in the GWAS catalog[a]

| SNP | HLA allele | $r^2$ | Function of SNP | Genes | Reported trait of SNP | Analyzed ethnicity | Reference |
|---|---|---|---|---|---|---|---|
| rs2860580 | HLA-A*11:01 | 0.87 | Unknown | — | Nasopharyngeal carcinoma | Southern Chinese descent | 31 |
| rs9263739 | HLA-C*12:02, HLA-B*52:01 | 0.94 | Intron | *CCHCR1* | Ulcerative colitis | Japanese | 11 |
| rs1265112 | HLA-C*04:01 | 0.83 | Intron | *CCHCR1* | Nevirapine-induced rash | HIV-infected Thai | 32 |
| rs4418214 | HLA-B*13:01, HLA-DRB1*12:02 | 1.0 | Unknown | — | HIV-1 control | Caucasian | 33 |
| rs2255221 | HLA-C*14:03, HLA-B*44:03 | 1.0 | Missense | *HCP5* | HIV-1 control | African-American | 33 |
| rs10484561 | HLA-DQB1*05:01 | 1.0 | Unknown | — | Follicular lymphoma | Caucasian | 34 |
| rs10484561 | HLA-DRB1*01:01 | 0.92 | Unknown | — | Follicular lymphoma | Caucasian | 34 |
| rs11752643 | HLA-DRB1*13:02 | 0.87 | Unknown | — | Coronary heart disease | Japanese | 12 |
| rs11752643 | HLA-DQB1*06:04 | 0.93 | Unknown | — | Coronary heart disease | Japanese | 12 |
| rs2281388 | HLA-DPB1*05:01 | 1.0 | Unknown | — | Graves disease | Chinese Han | 14 |

Abbreviations: GWAS, genome-wide association study; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; SNP, single-nucleotide polymorphism. [a]The GWAS catalog is based on the Table Browser of the UCSC Genome Bioinformatics database (GRCh37/hg19).

**Table 6.** List of HLA alleles and their tag haplotypes that include SNPs described in the GWAS catalog[a]

| Haplotype | HLA allele | $r^2$ between haplotype and HLA allele | SNPs in the haplotype | $r^2$ between SNP and HLA allele | Function of SNP | Genes | Reported trait of SNP | Analyzed ethnicity | Reference |
|---|---|---|---|---|---|---|---|---|---|
| rs4947296(C)-rs2442736(G) | HLA-C*14:02 | 1.0 | rs4947296 | 0.38 | Unknown | — | Graves disease | Chinese Han | 14 |
| | | | rs2442736 | 0.74 | Unknown | — | — | — | — |
| rs3815087(T)-rs9264885(T) | HLA-C*08:01 | 0.86 | rs3815087 | 0.38 | Untranslated | *PSORS1C1* | Stevens-Johnson syndrome and toxic epidermal necrolysis | Caucasian | 35 |
| | | | | | | | HIV-1 control | Caucasian | 36 |
| | | | rs9264885 | 0.79 | Unknown | — | — | — | — |
| rs2395148(A)-rs3763313(C)-rs9268979(T) | HLA-DRB1*08:03 | 0.92 | rs2395148 | 0.67 | Intron | *C6orf10* | Primary biliary cirrhosis | Caucasian | 37 |
| | | | | | | | Juvenile idiopathic arthritis | Caucasian | 38 |
| | | | rs3763313 | 0.25 | Near gene 5 | *BTNL2* | HIV-1 control | Caucasian | 36 |
| | | | rs9268979 | 0.22 | Unknown | — | — | — | — |
| rs3129888(C)-rs2187668(T) | HLA-DRB1*14:06 | 0.86 | rs3129888 | 0.48 | Intron | *HLA-DRA* | — | — | — |
| | | | rs2187668 | 0.48 | Intron | *HLA-DQA1* | Anti-dsDNA-positive systemic lupus erythematosus | Caucasian | 39 |
| | | | | | | | Idiopathic membranous nephropathy | Caucasian | 40 |
| | | | | | | | Immunoglobulin A deficiency | Caucasian | 41 |
| | | | | | | | Celiac disease | Caucasian | 42 |
| | | | | | | | Celiac disease | Caucasian | 43 |
| | | | | | | | Systemic lupus erythematosus | Caucasian | 44 |
| rs9272219(T)-rs4538747(T) | HLA-DQB1*03:01 | 0.85 | rs9272219 | 0.70 | Unknown | — | Rheumatoid arthritis | Caucasian | 45 |
| | | | | | | | Schizophrenia | Caucasian, African-American | 46 |
| | | | rs4538747 | 0.34 | Unknown | — | — | — | — |
| rs987870(C)-rs2855430(A) | HLA-DPB1*09:01 | 0.82 | rs987870 | 0.69 | Intron | *HLA-DPA1* | Pediatric asthma | Japanese | 13 |
| | | | | | | | Systemic sclerosis | Caucasian | 47 |
| | | | rs2855430 | 0.63 | Missense | *COL11A2* | — | — | — |

Abbreviations: dsDNA, double-stranded DNA; GWAS, genome-wide association study; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; SNP, single-nucleotide polymorphism. [a]The GWAS catalog is based on the Table Browser of the UCSC Genome Bioinformatics database (GRCh37/hg19).

which is located in *PRRC2A*, was in absolute LD with HLA-C*14:03 and HLA-B*44:03. A microsatellite analysis reported an association between the age-at-onset of insulin-dependent diabetes mellitus and *PRRC2A* in the Japanese population.[19] Although *PRRC2A* is located in the same region as the genes of tumor necrosis factor-α and tumor necrosis factor-β,[20,21] the effects of HLA-C*14:03 and


© 2012 Macmillan Publishers Limited

HLA-B*44:03 should be considered when surveying the causal factors of this disease in future studies.

The GWAS of Japanese pediatric asthma reported a significant association of rs987870, located in the intron of the *HLA-DPA1* locus, with this disease.[13] The significant association signals were distributed between the *HLA-DPB1*, *HLA-DPB2*, *COL11A2* and *RXRB* loci. The authors reported that HLA-DPA1*02:01, which was in strong LD with rs987870 and the HLA-DPB1*09:01 allele, was also significantly associated with the disease. Considering our data showing that rs987870 and the missense SNP (rs2855430) in the *COL11A2* region compose a tag haplotype for HLA-DPB1*09:01 (Table 6), it would be possible to highlight not only the effect of HLA-DPA1*02:01 and HLA-DPB1*09:01 but also that of *COL11A2*.

By comparison with a previous report,[2] the results of this study provide new information about the correlation between HLA alleles and SNPs in the Japanese population. Although the distributions of SNPs that show LD with HLA-A*33:03, A*31:01, C*01:02, C*14:03, B*52:01, DRB1*15:02 or DQB1*06:01, which are the relatively common HLA alleles in the Japanese population, were similar to the previous report, we also detected SNPs in strong LD with these HLA alleles that had not been previously described (Supplementary Figure 1). With respect to HLA-DRB1*13:02, the distribution of SNPs in LD and each LD status are somewhat different from the previous report. Our study showed that SNPs in strong LD with HLA-DRB1*13:02 are distributed in the region from 32 to ~33 Mb and that SNPs in LD ($0.2 \leqslant r^2 < 0.8$) are distributed across the entire extended MHC region, whereas the previous study showed that the SNPs in weak or moderate LD with this HLA allele are only distributed in two regions from 26 to 29 Mb and from 31 to 33 Mb. As we obtained similar results in the analysis of HLA-DQB1*06:04, which is in strong LD with DRB1*13:02 (Supplementary Figure 1), our data about the correlation between the SNPs and HLA-DRB1*13:02 and DQB1*06:04 could be applied to Japanese subjects.

Differences in the tag pattern between JPT, CEU and YRI were also indicated in the previous report.[2] For example, HLA-C*07:02 possessed many SNPs in moderate-to-strong LD in CEU and YRI across several Mb, whereas in JPT, the SNPs in strong LD were distributed in a narrow region near *HLA-C* locus.[2] On the other hand, tag SNPs for HLA-C*03:04 were not found in any population samples.[2] Consistent with the results, the SNPs in LD with HLA-C*07:02 are distributed within several kb of *HLA-C*, and HLA-C*03:04 does not possess tag SNP in our Japanese samples (Supplementary Figure 1). With respect to *HLA-DRB1*, we found that the SNPs in LD with DRB1*15:02 are distributed across 5.5 Mb in HLA region (Supplementary Figure 1), whereas in CEU, the SNPs in LD were distributed in a narrow region of this locus.[2] Thus, the tag pattern is likely to differ between populations even in a same HLA allele. This suggests that different SNPs could be associated with the same disease caused by a particular HLA allele in an analysis using different populations.

We utilized 92 Japanese samples and common SNPs; therefore, we could not analyze low-frequency HLA alleles. Recent advances in genomic analyses lead us to reaffirm the significance of the MHC region as a genetic factor in diseases. Genetic dissection of this region using not only common but also rare variants may be required for a comprehensive understanding of its roles in human disease susceptibility.

In conclusion, we identified tag SNPs and haplotypes for several HLA alleles of six HLA loci in the Japanese population. Our data will confer useful information for etiological studies of east Asian populations, specifically the Japanese population, focusing on both HLA and non-HLA genes in the MHC region.

## MATERIALS AND METHODS

### Subjects

Epstein–Barr virus-transformed B-cell lines derived from 92 healthy Japanese subjects were provided by the Riken Bioresource Center Cell Bank.[22] HLA typing was performed for the six loci (*HLA-A, C, B, DRB1, DQB1* and *DPB1*) by the Luminex microbead method (Luminex, Austin, TX, USA).

### SNP genotyping

SNP genotyping was carried out using the Illumina Human1M BeadChip (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. All subjects showed a genotyping call rate $>0.99$.

### Selection of SNPs and HLA alleles

SNPs that showed minor allele frequencies $<0.01$, call rates $<0.97$ (missed $>3$ samples) or Hardy-Weinberg equilibrium test *P*-values $<0.001$ were excluded from the analyses. HLA alleles with frequencies $<0.01$ were also excluded. A total of 82 HLA alleles (11, 11, 22, 18, 11 and 9 alleles in *HLA-A, C, B, DRB1, DQB1* and *DPB1*, respectively) and 6502 SNPs within the extended HLA region (the region from 26 to 34 Mb of chromosome 6) were subjected to the analyses. PLINK version 1.07[23] and R version 2.14.0[24] were used for the selection of SNPs and HLA alleles.

### Tag SNP and tag haplotype analysis

Initially, we calculated the pairwise LD between the HLA alleles and SNPs using Haploview.[25,26] Then, we assessed tag haplotypes for the HLA alleles that did not have SNPs in absolute LD. In this analysis, we chose SNPs in LD ($r^2>0.20$) with the HLA alleles and then carried out an aggressive search for tag haplotypes consisting of two or three SNPs by the 'Tagger' algorithm implemented in Haploview. The base position and function of the SNPs were based on the database of NCBI36/hg18. A total of 35 HLA alleles (4, 2, 13, 10, 4 and 2 alleles in *HLA-A, C, B, DRB1, DQB1* and *DPB1*, respectively) among 85 alleles do not possess tag SNPs or tag haplotypes ($r^2 \geqslant 0.8$). The SNPs reported to be associated with diseases were extracted from the GWAS catalog on the UCSC Genome Bioinformatics Browser (http://genome.ucsc.edu/).[27]

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

This work was supported by KAKENHI (22133003) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT).

## REFERENCES

1 Shiina T, Hosomichi K, Inoko H, Kulski JK. The HLA genomic loci map: expression, interaction, diversity and disease. *J Hum Genet* 2009; **54**: 15–39.
2 de Bakker PI, McVean G, Sabeti PC, Miretti MM, Green T, Marchini J *et al*. A high-resolution HLA and SNP haplotype map for disease association studies in the extended human MHC. *Nat Genet* 2006; **38**: 1166–1172.
3 Dong RP, Kimura A, Okubo R, Shinagawa H, Tamai H, Nishimura Y *et al*. HLA-A and DPB1 loci confer susceptibility to Graves' disease. *Hum Immunol* 1992; **35**: 165–172.
4 Richeldi L, Sorrentino R, Saltini C. HLA-DPB1 glutamate 69: a genetic marker of beryllium disease. *Science* 1993; **262**: 242–244.
5 Horiki T, Inoko H, Moriuchi J, Ichikawa Y, Arimori S. Combinations of HLA-DPB1 and HLA-DQB1 alleles determine susceptibility to early-onset myasthenia gravis in Japan. *Autoimmunity* 1994; **19**: 49–54.
6 Hori T, Kamikawaji N, Kimura A, Sone T, Komiyama N, Komiyama S *et al*. Japanese cedar pollinosis and HLA-DP5. *Tissue Antigens* 1996; **47**: 485–491.
7 Ito H, Yamasaki K, Kawano Y, Horiuchi I, Yun C, Nishimura Y *et al*. HLA-DP-associated susceptibility to the optico-spinal form of multiple sclerosis in the Japanese. *Tissue Antigens* 1998; **52**: 179–182.
8 Lv N, Dang A, Wang Z, Zheng D, Liu G. Association of susceptibility to Takayasu arteritis in Chinese Han patients with HLA-DPB1. *Hum Immunol* 2011; **72**: 893–896.
9 Ivansson EL, Juko-Pecirep I, Erlich HA, Gyllensten UB. Pathway-based analysis of genetic susceptibility to cervical cancer in situ: HLA-DPB1 affects risk in Swedish women. *Genes Immun* 2011; **12**: 605–614.
10 Saito S, Ota S, Yamada E, Inoko H, Ota M. Allele frequencies and haplotypic associations defined by allelic DNA typing at HLA class I and class II loci in the Japanese population. *Tissue Antigens* 2000; **56**: 522–529.
11 Asano K, Matsushita T, Umeno J, Hosono N, Takahashi A, Kawaguchi T *et al*. A genome-wide association study identifies three new susceptibility loci for ulcerative colitis in the Japanese population. *Nat Genet* 2009; **41**: 1325–1329.

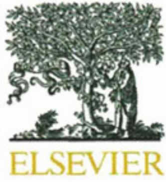
© 2012 Macmillan Publishers Limited

12 Takeuchi F, Yokota M, Yamamoto K, Nakashima E, Katsuya T, Asano H *et al.* Genome-wide association study of coronary artery disease in the Japanese. *Eur J Hum Genet* 2012; **20**: 333–340.
13 Noguchi E, Sakamoto H, Hirota T, Ochiai K, Imoto Y, Sakashita M *et al.* Genome-wide association study identifies HLA-DP as a susceptibility gene for pediatric asthma in Asian populations. *PLoS Genet* 2011; **7**: e1002170.
14 Chu X, Pan CM, Zhao SX, Liang J, Gao GQ, Zhang XM *et al.* A genome-wide association study identifies two new risk loci for Graves' disease. *Nat Genet* 2011; **43**: 897–901.
15 Aizawa H, Kinouchi Y, Negoro K, Nomura E, Imai G, Takahashi S *et al.* HLA-B is the best candidate of susceptibility genes in HLA for Japanese ulcerative colitis. *Tissue Antigens* 2009; **73**: 569–574.
16 Yoshida M, Kimura A, Katsuragi K, Numano F, Sasazuki T. DNA typing of HLA-B gene in Takayasu's arteritis. *Tissue Antigens* 1993; **42**: 87–90.
17 Elomaa O, Majuri I, Suomela S, Asumalahti K, Jiao H, Mirzaei Z *et al.* Transgenic mouse models support HCR as an effector gene in the PSORS1 locus. *Hum Mol Genet* 2004; **13**: 1551–1561.
18 Yates VM, Watkinson G, Kelman A. Further evidence for an association between psoriasis, Crohn's disease and ulcerative colitis. *Br J Dermatol* 1982; **106**: 323–330.
19 Hashimoto M, Nakamura N, Obayashi H, Kimura F, Moriwaki A, Hasegawa G *et al.* Genetic contribution of the BAT2 gene microsatellite polymorphism to the age-at-onset of insulin-dependent diabetes mellitus. *Hum Genet* 1999; **105**: 197–199.
20 Spies T, Blanck G, Bresnahan M, Sands J, Strominger JL. A new cluster of genes within the human major histocompatibility complex. *Science* 1989; **243**: 214–217.
21 Iris FJ, Bougueleret L, Prieur S, Caterina D, Primas G, Perrot V *et al.* Dense Alu clustering and a potential new member of the NF kappa B family within a 90 kilobase HLA class III segment. *Nat Genet* 1993; **3**: 137–145.
22 Iwakawa M, Goto M, Noda S, Sagara M, Yamada S, Yamamoto N *et al.* DNA repair capacity measured by high throughput alkaline comet assays in EBV-transformed cell lines and peripheral blood cells from cancer patients and healthy volunteers. *Mutat Res* 2005; **588**: 1–6.
23 Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007; **81**: 559–575.
24 R Development Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria, 2011 (http://www.R-project.org).
25 de Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D. Efficiency and power in genetic association studies. *Nat Genet* 2005; **37**: 1217–1223.
26 Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; **21**: 263–265.
27 Karolchik D, Hinrichs AS, Furey TS, Roskin KM, Sugnet CW, Haussler D *et al.* The UCSC Table Browser data retrieval tool. *Nucleic Acids Res* 2004; **32**(Database issue): D493–D496.
28 Takahashi M, Yasunami M, Kubota S, Tamai H, Kimura A. HLA-DPB1*0202 is associated with a predictor of good prognosis of Graves' disease in the Japanese. *Hum Immunol* 2006; **67**: 47–52.
29 Meguro A, Inoko H, Ota M, Katsuyama Y, Oka A, Okada E *et al.* Genetics of Behcet disease inside and outside the MHC. *Ann Rheum Dis* 2010; **69**: 747–754.
30 Matsuki K, Juji T, Tokunaga K, Takamizawa M, Maeda H, Soda M *et al.* HLA antigens in Japanese patients with myasthenia gravis. *J Clin Invest* 1990; **86**: 392–399.
31 Bei JX, Li Y, Jia WH, Feng BJ, Zhou G, Chen LZ *et al.* A genome-wide association study of nasopharyngeal carcinoma identifies three new susceptibility loci. *Nat Genet* 2010; **42**: 599–603.
32 Chantarangsu S, Mushiroda T, Mahasirimongkol S, Kiertiburanakul S, Sungkanuparph S, Manosuthi W *et al.* Genome-wide association study identifies variations in 6p21.3 associated with nevirapine-induced rash. *Clin Infect Dis* 2011; **53**: 341–348.
33 International HIV Controllers Study; Pereyra F, Jia X, McLaren PJ, Telenti A, de Bakker PI *et al.* The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. *Science* 2010; **330**: 1551–1557.
34 Conde L, Halperin E, Akers NK, Brown KM, Smedby KE, Rothman N *et al.* Genome-wide association study of follicular lymphoma identifies a risk locus at 6p21.32. *Nat Genet* 2010; **42**: 661–664.
35 Genin E, Schumacher M, Roujeau JC, Naldi L, Liss Y, Kazma R *et al.* Genome-wide association study of Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis in Europe. *Orphanet J Rare Dis* 2011; **6**: 52.
36 Fellay J, Ge D, Shianna KV, Colombo S, Ledergerber B, Cirulli ET *et al.* Common genetic variation and the control of HIV-1 in humans. *PLoS Genet* 2009; **5**: e1000791.
37 Hirschfield GM, Liu X, Xu C, Lu Y, Xie G, Lu Y *et al.* Primary biliary cirrhosis associated with HLA, IL12A, and IL12RB2 variants. *N Engl J Med* 2009; **360**: 2544–2555.
38 Behrens EM, Finkel TH, Bradfield JP, Kim CE, Linton L, Casalunovo T *et al.* Association of the TRAF1-C5 locus on chromosome 9 with juvenile idiopathic arthritis. *Arthritis Rheum* 2008; **58**: 2206–2207.
39 Chung SA, Taylor KE, Graham RR, Nititham J, Lee AT, Ortmann WA *et al.* Differential genetic associations for systemic lupus erythematosus based on anti-dsDNA autoantibody production. *PLoS Genet* 2011; **7**: e1001323.
40 Stanescu HC, Arcos-Burgos M, Medlar A, Bockenhauer D, Kottgen A, Dragomirescu L *et al.* Risk HLA-DQA1 and PLA(2)R1 alleles in idiopathic membranous nephropathy. *N Engl J Med* 2011; **364**: 616–626.
41 Ferreira RC, Pan-Hammarstrom Q, Graham RR, Gateva V, Fontan G, Lee AT *et al.* Association of IFIH1 and other autoimmunity risk alleles with selective IgA deficiency. *Nat Genet* 2010; **42**: 777–780.
42 Dubois PC, Trynka G, Franke L, Hunt KA, Romanos J, Curtotti A *et al.* Multiple common variants for celiac disease influencing immune gene expression. *Nat Genet* 2010; **42**: 295–302.
43 van Heel DA, Franke L, Hunt KA, Gwilliam R, Zhernakova A, Inouye M *et al.* A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21. *Nat Genet* 2007; **39**: 827–829.
44 Hom G, Graham RR, Modrek B, Taylor KE, Ortmann W, Garnier S *et al.* Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. *N Engl J Med* 2008; **358**: 900–909.
45 Eleftherohorinou H, Hoggart CJ, Wright VJ, Levin M, Coin LJ. Pathway-driven gene stability selection of two rheumatoid arthritis GWAS identifies and validates new susceptibility genes in receptor mediated signalling pathways. *Hum Mol Genet* 2011; **20**: 3494–3506.
46 Shi J, Levinson DF, Duan J, Sanders AR, Zheng Y, Pe'er I *et al.* Common variants on chromosome 6p22.1 are associated with schizophrenia. *Nature* 2009; **460**: 753–757.
47 Gorlova O, Martin JE, Rueda B, Koeleman BP, Ying J, Teruel M *et al.* Identification of novel genetic markers associated with clinical phenotypes of systemic sclerosis through a genome-wide association strategy. *PLoS Genet* 2011; **7**: e1002178.

Supplementary Information accompanies the paper on Genes and Immunity website (http://www.nature.com/gene)


© 2012 Macmillan Publishers Limited

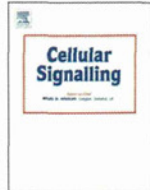
Cellular Signalling 25 (2013) 190–197



Contents lists available at SciVerse ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig



Review

# Nemo-like kinase, a multifaceted cell signaling regulator

Tohru Ishitani *, Shizuka Ishitani

*Division of Cell Regulation Systems, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

ARTICLE INFO

*Article history:*
Received 17 August 2012
Received in revised form 3 September 2012
Accepted 13 September 2012
Available online 18 September 2012

*Keywords:*
Nemo-like kinase
Phosphorylation
Wnt/β-catenin signaling

ABSTRACT

Nemo-like kinase (NLK) is an evolutionarily conserved MAP kinase-related kinase. Although NLK was originally identified as a *Drosophila* gene affecting cell movement during eye development, recent studies show that NLK also contributes to cell proliferation, differentiation, and morphological changes during early embryogenesis and nervous system development in vertebrates. In addition, NLK has been reported to be involved in the development of several human cancers. NLK is able to play a role in multiple processes due to its capacity to regulate a diverse array of signaling pathways, including the Wnt/β-catenin, Activin, IL-6, and Notch signaling pathways. Although the molecular mechanisms that regulate NLK activity remain unclear, our recent research has presented a new model for NLK activation. Here, we summarize the current understanding of the function and regulation of NLK and discuss the aspects of NLK regulation that remain to be resolved.

© 2012 Elsevier Inc. All rights reserved.

**Contents**

1. Introduction . . . 191
2. NLK as a cellular signaling modifier . . . 191
   2.1. Discovery of the molecular function of NLK . . . 191
   2.2. Dual and opposite effects of NLK-mediated LEF1 phosphorylation in Wnt/β-catenin signaling . . . 191
   2.3. Modulation of multiple transcriptional regulators by NLK . . . 192
   2.4. NLK activity near the plasma membrane . . . 194
3. Regulation of NLK . . . 194
   3.1. Molecular mechanisms that regulate NLK kinase activity . . . 194
   3.2. Regulation of NLK by microRNA . . . 195
   3.3. Mechanisms by which NLK selectively controls signaling pathways . . . 195
4. The roles of NLK in vertebrate development . . . 195
   4.1. The roles of NLK in nervous system development . . . 195
   4.2. The roles of NLK in non-neuronal tissues . . . 195
5. NLK expression and activity in cancer . . . 195
6. Kinases that share molecular functions with NLK . . . 196
7. NLK is a lithium chloride (LiCl)-sensitive kinase . . . 196
8. Conclusion . . . 196
Acknowledgment . . . 196
References . . . 196

*Abbreviations:* NLK, Nemo-like kinase; IL-6, intereukin-6; MAPK, mitogen activated protein kinase; ERK, extracellular-signal regulated kinase; Pro, proline; Ser, serine; Thr, threonine; *C. elegans*, *Caenorhabditis elegans*; POP-1, Posterior pharynx defect protein 1; LEF1, Lymphoid enhancer factor; TCF, T-cell factor; NARF, NLK associated RING finger protein; NPC, neural progenitor cell; HDAC1, histone deacetylase 1; Foxo, forkhead box O; STAT3, signal tranducer and activator of transcription 3; CPEB, cytoplasmic polyadenylation element-binding protein; Notch1-ICD, Notch1 intracellular domain; CSL, CBF-1, suppressor of hairless, LAG-1; SETDB1, SET domain bifurcated 1; PPAR-γ, peroxisome proliferator activated receptor-γ; BMP, bone morphogenetic protein; Eya, Eyes absent; Even-skipped, Eve; MAP1B, microtubule-associated protein 1B; NGF, nerve growth factor; TGF-β, transforming growth factor-β; MAPKK, MAPK kinase; Tyr, tyrosine; MAP3K, MAPK kinase kinase; Glu, glutamic acid; Cys, cysteine; TAK1, TGF-β-activated kinase 1; HIPK2, homeodomain interacting protein kinase 2; miRNA, microRNA; HCC, Hepatocellular carcinoma; ZIPK, Zipper-interacting protein kinase; GSK-3β, Glycogen synthase kinase-3β; LiCl, lithium chloride; IMPase, inositol monophosphatase; IPPase, inositol polyphosphate 1-phosphatase.

* Corresponding author. Tel.: +81 92 642 6789; fax: +81 92 642 6790.
*E-mail address:* tish@bioreg.kyushu-u.ac.jp (T. Ishitani).

0898-6568/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cellsig.2012.09.017

## 1. Introduction

Nemo-like kinase (NLK) was originally identified as *nemo*, a gene involved in cell motility (ommatidia rotation) during eye development in *Drosophila*. While wild-type flies form hexagonal-shaped ommatidia, *nemo* mutants form square-shaped ommatidia [1]. The name "nemo" derives from the Korean word for square. The vertebrate homolog of the *nemo* gene was discovered by Brott et al. in 1998, and was named "Nemo-like kinase" [2]. NLK is evolutionarily conserved from worms to humans (Fig. 1A). Invertebrate genomes possess only one NLK gene, while vertebrates have either one or two NLK genes. Vertebrate NLK proteins can be classified into two groups, type-I and type-II, by phylogenetic analysis (Fig. 1A) [3]. Amphibians and fish possess both type-I and type-II NLK, while mammals and chickens have only type-II NLK [3]. Type-II NLK, but not type-I NLK, contains histidine-rich amino-terminal and carboxyl-terminal conserved regions (Fig. 1B) [3]. Type-I NLK is known to be involved in early embryogenesis, in processes such as mesoderm induction [4,5]. However, type-II NLK knockout mice [6] and zebrafish *nlk2* knockdown embryos [3] display no early embryonic deficiencies. This suggests that the two NLKs have different functions in vertebrates. The differences in the biochemical properties of type-I NLK and type-II NLK remain unclear. In mice, the type-II NLK amino acid sequence is 54.5% similar and 41.7% identical to that of mouse mitogen activated protein kinase-1/extracellular-signal regulated kinase-2 (MAPK1/ERK2) [2]. Therefore, similar to MAPK1/ERK2, NLK is thought to function as a proline (Pro)-directed kinase, which phosphorylates proteins at a serine (Ser) or threonine (Thr) residue that is immediately preceding a Pro residue. In fact, NLK phosphorylates Lymphoid enhancer factor 1 (LEF1), a pivotal transcription factor in the Wnt/β-catenin signaling pathway, at the Thr and Ser residues of the Thr155-Pro156 and Ser166-Pro167 sequences [3,7]. However, the exact consensus target sequence of NLK has not been characterized. Over the past several years, evidence has emerged showing that NLK plays crucial roles in the regulation of diverse signaling pathways, including Wnt/β-catenin and Notch signaling pathways, and is involved in embryonic patterning, nervous system development, and cancer cell proliferation. Here, we discuss the function and regulation of NLK, with particular focus on vertebrate NLK.

## 2. NLK as a cellular signaling modifier

### 2.1. Discovery of the molecular function of NLK

In 1999, we and others found that the *C. elegans* mutant lacking endoderm, *lit-1*, possessed a mutation in a NLK homolog gene and that the gene product of *lit-1* inhibited nuclear localization of Posterior pharynx defect protein 1 (POP-1) by phosphorylating it during endoderm induction [8,9]. This was the first discovery of a molecular function of NLK. POP-1 is a homolog of the mammalian T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors that regulate Wnt/β-catenin signaling. Therefore, we hypothesized that mammalian NLK was involved in Wnt/β-catenin signaling. We found that mammalian NLK could phosphorylate vertebrate TCF/LEF transcription factors, including *Xenopus laevis* TCF7L1/TCF3 and human TCF7L2/TCF4 and LEF1. Moreover, the TCF7L2 proteins phosphorylated by NLK in the human embryonic kidney cell line HEK293 lacked DNA-binding activity in electron mobility shift assays [7,10] (Fig. 2A). In addition, NLK-mediated LEF1 phosphorylation inhibited binding of LEF1 to its target gene, the *Axin2* promoter, in HeLa cells [3]. Furthermore, we discovered that overexpression of NLK inhibited TCF/LEF-mediated transcription in both HeLa and HEK293 cells [3,7,10] (Fig. 2A). Yamada et al. also reported that NLK promotes NARF (NLK associated RING finger protein)-mediated ubiquitination and the subsequent proteasomal degradation of TCF7L2 and LEF1 [11]. Thus, NLK is considered a negative regulator of TCF/LEF in vertebrates.

### 2.2. Dual and opposite effects of NLK-mediated LEF1 phosphorylation in Wnt/β-catenin signaling

Interestingly, LIT-1 functions as a positive regulator of POP-1 during the fate specification of gonadal precursor cells in *C. elegans* [12–14]. Recently, we reported that vertebrate NLK also functions as a positive regulator of LEF1 in neural progenitor cells (NPCs) [3] (Fig. 2B). In the NPC-like mammalian cell lines, rat pheochromocytoma tumor PC12 cells and mouse neuroblastoma neuro-2a cells, NLK-mediated LEF1 phosphorylation at Thr-155 and Ser-166 induced

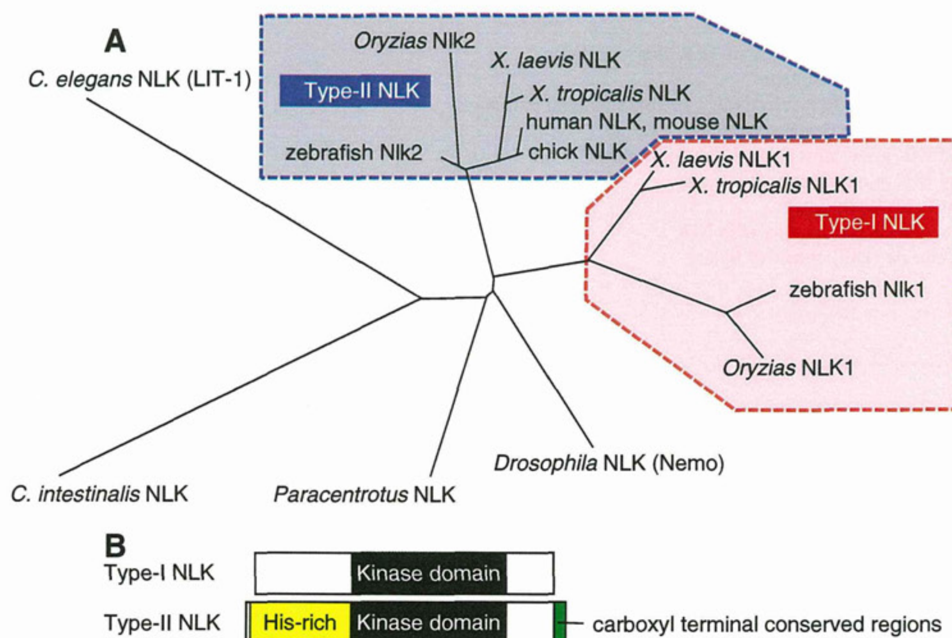



**Fig. 1.** NLK family proteins. (A) Phylogenetic analysis of NLK homologs by comparison of amino acid sequences. Vertebrate type-I and type-II NLKs are shown in blue and red, respectively. (B) Schematic diagrams of Type-I and Type-II NLKs. Note that type-II NLKs, but not type-I NLKs, have conserved histidine-rich (His-rich) and carboxyl-terminal regions, which are indicated by yellow and green boxes, respectively.

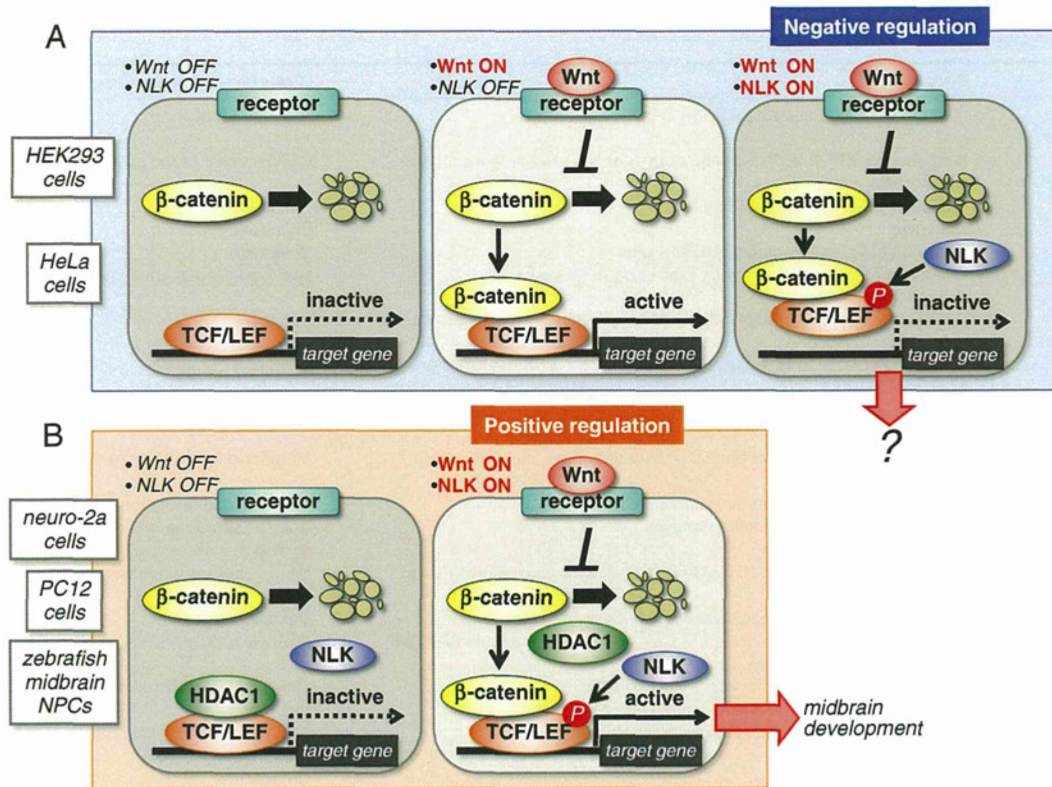



**Fig. 2.** Cell context-dependent positive and negative regulation of Wnt/β-catenin signaling by NLK. Under unstimulated conditions, β-catenin is destabilized and TCF/LEF represses target gene expression in all cell types (left panels in A and B). In neuro-2a and PC12 cells and zebrafish midbrain NPCs, but not in HEK293 or HeLa cells, HDAC1 strongly inhibits LEF1 transcriptional activity (left panels in A and B). The binding of Wnt ligand to the receptor induces the stabilization of β-catenin in all cell types (middle panel in A and right panel in B). In HEK293 and HeLa cells, stabilized β-catenin forms a complex with TCF/LEF, resulting in the activation of target gene expression (middle panel in A). Overexpression of NLK inhibits Wnt ligand-induced target gene expression by blocking its DNA-binding activity (right panel in A). In neuro-2a and PC12 cells and zebrafish midbrain NPCs, Wnt ligand-activated Dvl promotes Wnt/β-catenin signaling target gene expression via formation of the β-catenin-LEF1 complex and phosphorylation of LEF1 by NLK and the consequent dissociation of LEF1 from HDAC1 (right panel in B). NLK-mediated LEF1 positive regulation contributes to midbrain development (A), while the physiological roles of the negative regulation of LEF1 by NLK have not been determined (A).

dissociation of LEF1 from histone deacetylase HDAC1, thereby promoting transcription activation. The Lef1 phosphorylation did not affect the binding of LEF1 to its target gene promoter. Signaling induced by Wnt-3a, a member of the Wnt-1 family of Wnt/β-catenin ligands, promoted NLK kinase activation, LEF1 phosphorylation and HDAC1 dissociation from LEF1. In agreement with this, knockdown of NLK attenuated transcriptional activation induced by Wnt-3a. Furthermore, inhibition of Nlk2, a zebrafish type-II NLK, decreased LEF1 phosphorylation, LEF1-mediated gene expression, and cell proliferation in the presumptive midbrain, resulting in a reduction in midbrain tectum size. The phenotype caused by Nlk2 knockdown was suppressed by expression of a LEF1 mutant that mimics the constitutively phosphorylated state or by co-knockdown of HDAC1. Knockdown of Wnt1, which is expressed in the presumptive midbrain, resulted in phenotypes similar to those caused by Nlk2 knockdown [3]. These findings suggest that Nlk2 is required for Wnt/β-catenin signaling through LEF1 phosphorylation and for the abrogation of HDAC1-mediated LEF1 inhibition in zebrafish midbrain. Thus, NLK-mediated LEF1 phosphorylation has a dual and opposite effect in Wnt/β-catenin signaling. The first is the inhibition of LEF1 DNA-binding activity in HeLa and HEK293 cells, and the second is the release of HDAC1 from the LEF1 transcriptional complex in NPCs (Fig. 2 and Table 1).

It is unclear why NLK is required for activating TCF/LEF-mediated transcription in the zebrafish midbrain and NPC-like mammalian cell lines but not in HEK293 and HeLa cells. Interestingly, we found that the inhibitory effect of HDAC1 on LEF1-mediated transcription is relatively weak in HEK293 and HeLa cells [3]. Differences in the inhibitory effect of HDAC1 in different cell types may explain the seemingly contradictory effects of NLK mentioned above. The molecular mechanisms underlying these dual and opposite roles of NLK in Wnt/β-catenin signaling have yet to be characterized. The phosphorylation level of LEF1 in HeLa cells is much higher than that of LEF1 in neuro-2a and PC12 cells [3], suggesting that perhaps additional phosphorylation results in negative regulation of LEF1. Based on the results discussed above, comparison of the TCF/LEF-binding proteins and TCF/LEF phosphorylation sites in these cell lines may help to elucidate this mechanism.

### 2.3. Modulation of multiple transcriptional regulators by NLK

Over the past decade, a number of transcriptional regulators have been identified as substrates of vertebrate NLK. c-Myb is a transcription factor that regulates hematopoietic stem cell proliferation and differentiation [15,16]. In CV-1 cells, NLK phosphorylates c-Myb, resulting in its degradation [17] (Table 1). Another member of the Myb gene family, A-Myb, can be also phosphorylated by NLK. Phosphorylation of A-Myb inhibits its association with the transcriptional coactivator CBP, but does not induce A-Myb degradation [18] (Table 1). The Foxo family of transcription factors modulates the expression of target genes involved in apoptosis, the cell cycle, stress response, longevity, DNA repair, and glucose metabolism [19]. Of the members of the Foxo family, Foxo1, Foxo3a, and Foxo4 are phosphorylated by NLK. NLK-mediated Foxo1 phosphorylation has been shown to inhibit Foxo1-mediated transcription by promoting its nuclear export in the CV-1 cell derivative cell line, Cos-1 [20,21] (Table 1). However, the physiological roles of c-Myb and Foxo phosphorylation remain undefined.

**Table 1**
Targets of NLK family of protein kinases.

| Targets | Direct effects | Effects on cells and/or tissues *in vivo* |
|---|---|---|
| *C. elegans* POP-1 (a homolog of TCF/LEF) | Inhibition of POP-1 nuclear localization in *C. elegans* embryos | Endoderm induction |
| *C. elegans* POP-1 (a homolog of TCF/LEF) | Promotion of POP-1 mediated gene expression in *C. elegans* gonadal precursor cells | Establishment of the proximal–distal axis of the gonad |
| Human TCF7L2 | Inhibition of TCF7L2 DNA-binding *in vitro* | Unknown |
| *Xenopus* TCF7L1 | Unknown | Unknown |
| Human LEF1 | Inhibition of LEF1 DNA-binding in HeLa cells | Unknown |
| Human/mouse/ zebrafish LEF1 | Promotion of LEF1-mediated gene expression in neuro-2a and PC12 cells and zebrafish midbrain. | Size expansion on midbrain tectum |
| Mouse c-Myb | Degradation of c-Myb and inhibition of c-Myb-mediated gene expression in CV-1 cells | Unknown |
| A-Myb | Dissociation of CBP from A-Myb and inhibition of A-Myb-mediated gene expression in CV-1 cells | Unknown |
| Foxo1 | Export of Foxo1 from nucleus and inhibition of Foxo1-mediated gene expression | Unknown |
| *Xenopus*/mouse STAT3 | Promotion of STAT3 transcriptional activity in *Xenopus* embryos and in HepG2 cells | Mesoderm induction in *Xenopus* embryod |
| *Xenopus* MEF2A | Promotion of MEF2Aactivity in *Xenopus* embryos | Anterior head formation |
| *Xenopus* CPEB | Degradation of CPEB in *Xenopus* embryos | Oocyte maturation in the absence of progesterone (in NLK overexpressed oocytes) |
| Human/mouse/ zebrafish Notch1 | Inhibition of the active Notch1-ICD transcriptional complex formation in neuro-2a cells | Neurogenesis in zebrafish neural plate |
| SETDB1 | Inhibition of PRAR-γ-mediated transactivation in ST2 cells | Unknown |
| *Drosophila* Mad | Inhibition of Mad nuclear localization and consequent suppression of BMP signaling in *Drosophila* wing disc | *Drosophila* wing development |
| *Drosophila* Period | Promotion of Period protein stability in *Drosophila* | Coordination of circadian rhythm |
| *Drosophila* Eya | Promotion of Eya mediated gene expression in wing disc | Unknown |
| *Drosophila* Eve | Enhancement of Eve-mediated gene repression | Patterning of *Drosophila* early embryos |
| Rat MAP1B | Unknown | Unknown |
| Rat Paxillin | Unknown | Unknown |

During early embryogenesis in *X. laevis*, the type-I NLK, xNLK1, phosphorylates two transcription factors, STAT3 and MEF2A. Phosphorylation of STAT3 enhances its transcriptional activity and contributes to mesoderm induction [4] (Table 1), while MEF2A phosphorylation is involved in anterior head formation [22] (Table 1). Ota et al. also reported that overexpression of xNLK1 in *Xenopus* oocytes could induce the phosphorylation of key regulators of translational control, including Pumilio1, Pumilio2, and cytoplasmic polyadenylation element-binding protein (CPEB), as well as CPEB degradation and oocyte maturation in the absence of progesterone [23] (Table 1). However, they failed to demonstrate the physiological role of xNLK1 in oocyte maturation by xNLK1 knockdown.

We recently identified Notch1 intracellular domain (Notch1-ICD), a critical transcriptional regulator of Notch signaling, as an NLK substrate [24]. NLK phosphorylates Notch1-ICD at seven Ser residues on conserved Ser-Pro motifs *in vitro* and in neuro-2a cells. Overexpression of NLK inhibited Notch1-ICD-mediated gene expression via Notch1 phosphorylation in HEK293, HeLa, neuro-2a, and PC12 cells and in the colorectal cancer cell line SW480, and knockdown of NLK with RNAi enhanced Notch1-ICD-mediated gene expression in neuro-2a and SW480 cells. NLK-mediated Notch1-ICD phosphorylation prevented complex formation of Notch1-ICD with the DNA-binding factor CSL (CBF-1, suppressor of hairless, and LAG-1) and a transcriptional coactivator of the Mastermind family in neuro-2a cells and *in vitro* [24] (Table 1). These findings suggest that NLK is a negative regulator of Notch signaling. We also found that knockdown of zebrafish type-I NLK (Nlk1) enhanced Notch signaling and inhibited neurogenesis in the zebrafish neural plate. In addition, the effects of a phosphorylation-resistant Notch1-ICD mutant on Notch signaling activation and neurogenesis suppression in zebrafish neural plate were stronger than those of wild-type Notch1-ICD, while the effects of a Notch1-ICD mutant that mimics the phosphorylated state were weaker [24]. This suggests that NLK-mediated regulation of Notch signaling is essential for proper neurogenesis in the zebrafish neural plate [24]. More recently, it was reported that inhibition of NLK-mediated Notch signaling contributes to natural killer cell development *in vitro* [25].

NLK is also reported to participate in chromatin regulation. NLK regulates chromatin downstream of Wnt-5a signaling by phosphorylating SET domain bifurcated 1 (SETDB1), a methyltransferase of Histone-H3 [26] (Table 1). Wnt-5a signaling activates NLK-mediated SETDB1 phosphorylation, which suppresses peroxisome proliferator activated receptor-γ (PPAR-γ)-mediated transactivation in the mouse bone marrow-derived stromal cell line, ST2 [26]. Although it has been shown that Wnt-5a induces osteoblastogenesis through attenuating PPAR-γ-induced adipogenesis in ST2 cells [26], the relationship between SETDB1 phosphorylation and osteoblastogenesis and adipogenesis remains unclear.

Recent studies in *Drosophila* reveal novel nuclear functions of NLK. During early embryogenesis, Nemo phosphorylates Even-skipped (Eve) [27], a pair-rule gene that encodes a homeobox transcription repressor and plays essential roles in establishing metameric segmentation [28]. Nemo-mediated phosphorylation of Eve enhances Eve-mediated suppression of gene expression and contributes to embryonic patterning [27]. During wing development, Nemo blocks bone morphogenetic protein (BMP) signaling by phosphorylating Mad [29], the *Drosophila* homolog of the BMP signaling transcription factor, Smad [30], which inhibits its nuclear accumulation [29] (Table 1). During eye formation, Nemo phosphorylates Eyes absent (Eya), a transcriptional regulator essential for eye specification in a variety of organisms [31]. This phosphorylation of Eya enhances its transactivation function [32] (Table 1). Nemo also regulates circadian rhythms [33] through its role as a transcriptional regulator of Period, the circadian clock component that behaves as the primary phospho-timer [34]. Nemo-mediated phosphorylation promotes the stability of Period and coordinates circadian rhythm [33] (Table 1). All of these Nemo substrates and their molecular functions and regulation are highly conserved in vertebrates, suggesting that NLK may regulate these proteins in a similar manner.

### 2.4. NLK activity near the plasma membrane

In many mammalian cells, exogenous NLK proteins are localized primarily to the nucleus [2,35], suggesting that this is the primary site of NLK function. However, we discovered that in rat PC12 and mouse neuro-2a cells, endogenous NLK localizes to the perinuclear region, including the Golgi apparatus, and not to the nucleus. In addition, stimulation with nerve growth factor (NGF) promoted not only the translocation of endogenous NLK into the nucleus and leading edges of the cell, but also induced the enzymatic activation of NLK [36]. These findings suggest that NLK functions both in the nucleus and near the plasma membrane. In agreement with this, we recently found that following treatment with NGF, NLK phosphorylated the focal adhesion adaptor protein paxillin at Ser-126 and the microtubule-associated protein 1B (MAP1B) at the leading edge of PC12 cells [36]. NLK knockdown reduces the phosphorylation of paxillin Ser-126 and MAP1B, actin network formation in the leading edges, and neurite growth in NGF-treated PC12 cells [36]. Although the physiological roles of these phosphorylations remain unclear, these results suggest that NLK may contribute to the formation and extension of neurites in differentiating neurons *in vivo*. Invertebrate NLK can also localize to the plasma membrane. *C. elegans* LIT-1 localizes to the cell cortex during asymmetric cell division [37], and *Drosophila* Nemo localizes to the plasma membrane during ommatidial rotation [38]. These observations support the theory that NLK family proteins function both in the nucleus and near the plasma membrane. Further investigation of the functions of NLK at the plasma membrane will be important.

## 3. Regulation of NLK

### 3.1. Molecular mechanisms that regulate NLK kinase activity

A variety of extracellular signals can stimulate the activation of NLK kinase activity. Endogenous NLK can be activated in Wnt-3a-treated PC12 cells [3], NGF-treated PC12 cells [36], Activin A-treated HEK293 cells [4], and Wnt-5a-overexpressing HEK293 cells [39]. Treatment with IL-6 or transforming growth factor-β (TGF-β) can activate exogenous NLK in the human hepatocellular carcinoma cell line HepG2 [40]. However, the molecular mechanisms that regulate NLK kinase activity remain unclear. As described above, the amino acid sequence of NLK is similar to that of ERK2 MAPK [2]. The canonical MAPKs, including ERK2, possess a characteristic MAPK-activating phosphorylation sequence, Thr-Xxx-Tyr (TXY), in the activation loop just upstream of the conserved kinase domain VIII [41]. MAPKs are activated by the phosphorylation of Thr and Tyr residues in the TXY motif by a family of dual-specificity MAPK kinases (MAPKKs) [41–43]. MAPKK-mediated ERK2 phosphorylation not only stimulates the kinase activity of ERK2 but also induces dimerization (Fig. 3A) [44–46]. Thus, the activity of canonical MAPKs is tightly regulated by their upstream kinases. However, we and others showed previously that overexpression of NLK alone is sufficient for activation of its kinase activity in mammalian cells [2,35], suggesting that when overexpressed, NLK can become active without being phosphorylated by upstream kinases. Interestingly, NLK family proteins do not possess a TXY motif in their activation loop. Instead they possess the sequence Thr-Xxx-Glu (TQE) at an analogous site [2]. Glutamic acid, a negatively charged amino acid, can mimic the phosphorylated amino acid. Taken together, these observations suggest that phosphorylation of Thr-286 in the TQE sequence may be involved in NLK activation.

Recently, we discovered that NLK can become activated by autophosphorylating Thr-286 when overexpressed [35]. Size exclusion gel-filtration chromatography revealed that exogenous NLK expressed in HEK293 cells forms homo-dimers. Upon homo-dimer formation, NLK autophosphorylates Thr-286 in a *trans* manner within the dimer. Substitution of Thr-286 with a valine residue or phosphatase treatment strongly reduced NLK kinase activity. Mutation of NLK at Cys-425, which corresponds to the defect in the *C. elegans* mutant *lit-1*, prevented

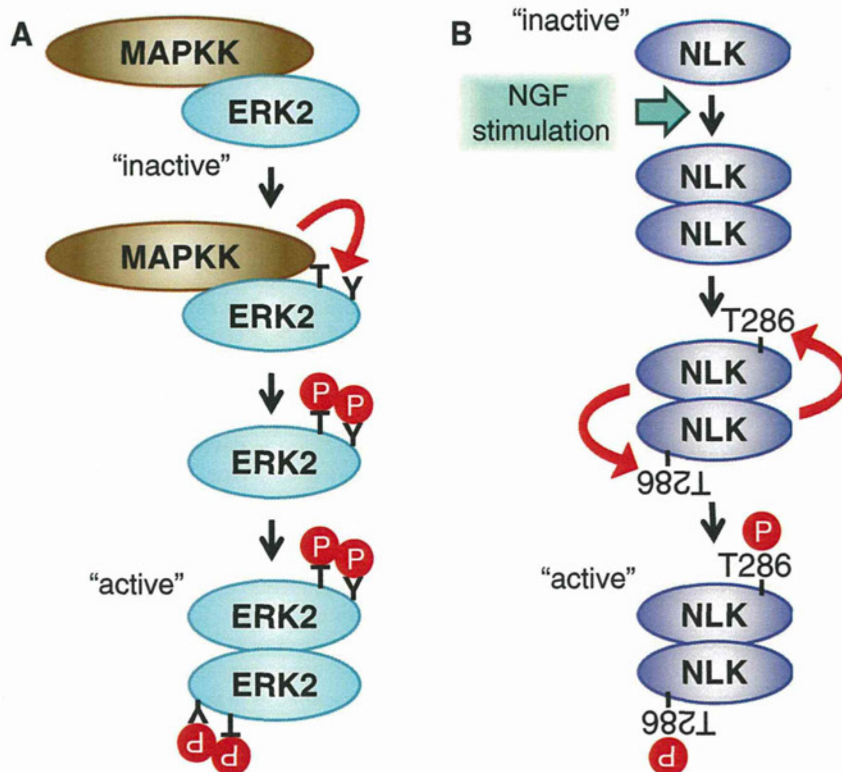



**Fig. 3.** Model comparing the known mechanism of ERK2 MAPK activation with NLK autophosphorylation/autoactivation. ERK2 MAPK is activated by phosphorylation of Thr and Tyr residues in the TXY motif by MAPKKs. This ERK2 phosphorylation not only activates the kinase activity of ERK2 but also induces its dimerization (A). NGF signaling induces NLK dimerization and consequent Thr-286 autophosphorylation of NLK (B).

NLK dimerization, rendering NLK defective in kinase activity. By contrast, the addition of exogenous NGF induced dimerization and autophosphorylation of Thr-286 in endogenous NLK proteins [35]. These findings suggest that NLK dimerization and the consequent NLK autophosphorylation of Thr-286 is essential for NLK activation (Fig. 3B).

Although the molecular mechanisms that regulate NLK dimerization remain unclear, some kinases that activate NLK have been identified. During endoderm induction in *C. elegans*, the MAPK kinase kinase (MAP3K)-related kinase MOM-4 activates LIT-1 [8,47]. Activation of the vertebrate MOM-4 homolog, TGF-β-activated kinase 1 (TAK1), can promote NLK autophosphorylation activity [10] and is essential for Wnt-5a-induced NLK activation in HEK293 cells [39]. Interestingly, an interaction between TAK1 and NLK was not detected by yeast two-hybrid (unpublished data), suggesting that TAK1 does not directly bind to NLK. Kanei-Ishii et al. reported that homeodomain-interacting protein kinase 2 (HIPK2) is directly phosphorylated by TAK1 and binds to and activates NLK [17]. Ohnishi et al. reported that p38, a well-known downstream MAPK of TAK1 MAP3K signaling, directly interacts with NLK and regulates its kinase activity [48]. However, neither HIPK2 nor p38 phosphorylate NLK at Thr-286 [17,48]. It will be interesting to determine whether phosphorylation by HIPK2 or p38 is involved in NLK dimerization.

### 3.2. Regulation of NLK by microRNA

Several recent reports have shown that NLK expression is regulated by microRNA (miRNA). During natural killer cell development, the levels of miRNA-181a and miRNA-181b transcripts increase [25]. miRNA-181a and miRNA-181b reduce the levels of NLK protein and abrogate NLK-mediated negative regulation of Notch signaling, thus promoting human natural killer cell development *in vitro* [25]. miRNA-181a, miRNA-181b, miRNA-181c, and miRNA-181d also negatively regulate the expression level of NLK protein in hepatocellular carcinoma (HCC) cell lines [49]. miRNA-181 family members may control NLK expression in a variety of tissues.

### 3.3. Mechanisms by which NLK selectively controls signaling pathways

As described above, NLK phosphorylates and regulates a variety of signaling molecules. It is noteworthy that, in several cases, the phenotypes of cell- or stage-specific NLK knockout/knockdown animals depend on the specific NLK target. For example, the midbrain tectum size reduction phenotype in zebrafish Nlk2-knockdown embryos was completely reversed by expression of a phosphorylation-state mimicking mutant of LEF1 [3]. Knockdown of Nlk1 failed to induce the increased-neuron phenotype in the neural plate of Notch-knockdown zebrafish embryos [24]. These observations suggest that NLK targets may be selected in a cell context and/or condition-dependent manner. However, little is known about NLK target selection, although some evidence exists suggesting that STAT3 and Zipper-interacting protein kinase (ZIPK) may contribute to this system. STAT3 forms a complex with both NLK and its activator TAK1 and specifically promotes IL-6 signaling-induced activation of the TAK1-NLK pathway and the consequent phosphorylation of STAT3 by NLK [40]. ZIPK interferes with the interaction of NLK with its substrate TCF7L2 in HEK293 cells [50], suggesting that ZIPK may selectively block NLK-mediated regulation of Wnt/β-catenin signaling. To fully understand the *in vivo* roles of NLK, the mechanism by which NLK targets are selected must be elucidated.

## 4. The roles of NLK in vertebrate development

### 4.1. The roles of NLK in nervous system development

Vertebrate NLK family genes are highly expressed in neural tissues [2,3,5,48] and play crucial roles in nervous system development. *X. laevis* type-I NLK, xNLK1, regulates the development of anterior neural structures by regulating the activity of MEF2A [22]. Zebrafish type-I NLK, Nlk1, controls brain anterior/posterior patterning, possibly in cooperation with Wnt/β-catenin signaling [5], and promotes primary neurogenesis in the neural plate by inhibiting Notch signaling [24]. Zebrafish type-II NLK, Nlk2, promotes neural progenitor cell proliferation in the presumptive midbrain by positively regulating Wnt/β-catenin signaling, and contributes to the size expansion of the midbrain tectum [3]. Mammalian type-II NLK also contributes to NGF-induced neurite outgrowth in PC12 cells. Thus, NLK is involved in a variety of processes during nervous system development. Interestingly, Kortenjann et al. observed that type-II NLK -deficient mice suffer from various neurological abnormalities, including cerebellar ataxia [6]; however, a detailed phenotype was not provided. Future analysis of the effects of knockdown of NLK on neuronal development may reveal whether the neuronal functions of type-II-NLK are conserved in vertebrates.

### 4.2. The roles of NLK in non-neuronal tissues

NLK has also been shown to regulate bone formation. Nifuji et al. reported that overexpression of NLK, but not a kinase-inactive mutant of NLK, suppressed the expression of bone markers in primary calvarial osteoblasts and the bone marrow stromal cell line ST2, while knockdown of NLK with siRNA enhanced bone marker expression [51]. Zanotti and Canalis showed that knockdown of NLK in ST2 cells promoted BMP and Wnt/β-catenin signaling [52], pathways that play a central role in osteoblastic differentiation [53]. These observations suggest that NLK negatively regulates osteoblastic differentiation by blocking BMP and Wnt/β-catenin signaling. However, the mechanism by which NLK regulates BMP and Wnt/β-catenin signaling and the molecular link between this regulation and osteoblastic differentiation remain unknown. Conversely, Kortenjann et al. reported that osteogenesis was unaffected in NLK knockout mice, although these mice were only maintained until 4 weeks after birth [6]. Further investigation of osteoblastgenesis and osteogenesis in NLK knockout mice at later stages will be important.

In some NLK-deficient mice at 3–4 weeks after birth, adipogenesis in bone marrow was enhanced, while the number of hematopoietic cells was reduced [6], suggesting that NLK is involved in adipogenesis and hematopoiesis. As described above, NLK suppresses PPAR-γ-mediated transactivation, a process that positively regulates adipogenesis [26]. This may explain the increased adipogenesis observed in NLK null mice, although the exact molecular mechanisms by which NLK regulates adipogenesis and hematopoiesis have not been clarified.

## 5. NLK expression and activity in cancer

During the past several years, a number of studies have reported a correlation between NLK expression and activity and cancer development. Jung et al. discovered that expression of NLK is up-regulated in HCCs and showed that NLK positively regulates the expression of cyclinD1, a core component of cell cycle regulation [54]. It is well-known that the development and progression of HCCs are associated with increased Wnt/β-catenin signaling [55]. Interestingly, NLK promotes cyclinD1 expression by positively regulating Wnt/β-catenin signaling in neuro-2a cells [3]. NLK might contribute to the growth of HCC by enhancing Wnt/β-catenin signaling activity. However, NLK appears to function as a tumor suppressor in prostate cancer and glioma. Emami et al. found that NLK expression is decreased during prostate cancer progression and demonstrated that NLK inhibits androgen receptor (AR) expression and subsequent AR-mediated transcription and promotes apoptosis in prostate cancer cell lines [56]. Cui et al. reported that NLK expression levels are higher in human glioma tissues from lower grade tumors and that the survival rate of patients with gliomas expressing low levels of NLK is significantly shorter than that of patients with gliomas expressing high levels of NLK [57]. It will be important to clarify the direct target (substrate) of NLK activity and the mechanisms that regulate NLK expression in these cancer cells.

## 6. Kinases that share molecular functions with NLK

Zebrafish Nlk1 and xNLK1 are involved in the processes of early embryogenesis, such as mesoderm induction [4,5]. Interestingly, type-II NLK knockout mice do not display early embryonic deficiencies [6], even though mammals do not possess type-I NLK [3]. This may be explained by the potential for other genes to compensate for the loss of type-I NLK activity and maintain early embryogenesis. Recently, HIPK2 was found to regulate Wnt/β-catenin signaling in a manner similar to NLK [58,59]. Hikasa et al. reported that *Xenopus* HIPK2 was able to phosphorylate LEF1, TCF7L1, and TCF7L2, but not TCF7, *in vitro*, and that overexpression of HIPK2 reduced the binding of LEF1 and TCF7L1 to the *vent2* gene promoter in *Xenopus* embryos [58,59]. These HIPK2 functions resemble those of NLK in HeLa and HEK293 cells. We have also reported that, similar to NLK, HIPK2 promoted Wnt/β-catenin signaling by phosphorylating LEF1 at Thr-155 and Ser-166 in neuro-2a cells [3]. Thus, NLK and HIPK2 may cooperate to regulate Wnt/β-catenin signaling activity in vertebrates. Glycogen synthase kinase-3β (GSK-3β) also shares molecular functions with NLK. GSK-3β can phosphorylate paxillin at Ser-126 and MAP1B and contributes to NGF-induced neurite outgrowth [60–63]. In addition, GSK-3β negatively regulates not only Wnt/β-catenin signaling [64,65] but also Notch signaling [66,67]. GSK-3β may function cooperatively and/or redundantly with NLK to regulate several signaling processes.

## 7. NLK is a lithium chloride (LiCl)-sensitive kinase

We recently found that NLK is sensitive to LiCl [36], which is well-known to inhibit GSK-3, but not specifically [68–70]. LiCl treatment in PC12 cells inhibited NGF-induced endogenous NLK activation. In addition, NLK immunoprecipitated from mammalian cells was unable to undergo autophosphorylation in the presence of LiCl [36]. These observations suggest that LiCl directly inhibits the kinase activity of NLK.

LiCl interferes with several signaling processes, including Wnt/β-catenin signaling- and Notch signaling-mediated gene expression and NGF-signaling-induced neurite outgrowth [66,67,71–73], which are also regulated by NLK [10,24,36]. In addition, LiCl is used to treat neurological diseases, such as bipolar disorder [74]. Previous studies have identified a number of LiCl target enzymes, including GSK-3β, inositol monophosphatase (IMPase), and inositol polyphosphate 1-phosphatase (IPPase) [75]. Despite the therapeutic benefits of lithium, its precise mechanism of action remains elusive. Berridge et al. proposed that depletion of inositol caused by lithium-mediated inhibition of IPPase and IMPase activities leads to mood stabilization [76]. Recently, Beaulieu et al. showed that lithium exerts its behavioral effects in mice by activating the Ser/Thr kinase Akt, which directly inhibits GSK-3β activity, by disrupting a signaling complex composed of Akt, β-arrestin 2, and protein phosphatase 2A [77,78]. Since NLK is highly expressed in the brain, the behavioral effects induced by lithium might also be mediated by changes in NLK activity. Reexamination of LiCl-sensitive events in light of the role for NLK might lead to novel insights not only into the therapeutic mechanism of lithium activity but also into the physiological roles of NLK.

## 8. Conclusion

NLK was first identified as a gene that contributes to cell movement in *Drosophila*. Conversely, NLK in *C. elegans* was isolated as a gene required for cell fate decisions. These differences in functionality predicted that NLK may play multiple roles at both the molecular and cellular levels. However, studies on NLK to date show that the roles of NLK are more diverse than had been expected. NLK controls gene expression and cytoskeletal architecture by phosphorylating and regulating a variety of cell signaling components and cytoskeleton-associated proteins. However, it is still not clear how the specific substrates are targeted and regulated by NLK under different conditions. It is also expected that NLK is able to regulate multiple targets simultaneously to coordinate the activities of multiple signaling pathways in response to specific stimuli. These issues are the focus of future studies. In both basic biology fields and medical research fields, such as cell fate control and cancer research, NLK is emerging as a remarkable and multifaceted gene. Although specific chemical inhibitors of NLK have not yet been developed, they are likely to be a useful tool in the treatment of multiple diseases.

## Acknowledgment

This research was supported by a Health Labour Sciences Research Grant (T.I.), Kaibara Morikazu Medical Science Promotion Foundation (T.I.), Kurata Memorial Hitachi Science and Technology Foundation (T.I.), Nakajima Foundation (T.I.), Kyushu University P&P (S.I.), Hayashi Memorial Foundation for Female Natural Scientists (S.I.), and the Grants-in-Aid for Scientific Research programs in Japan (T.I. and S.I.).

## References

[1] K.W. Choi, S. Benzer, Cell 78 (1994) 125–136.
[2] B.K. Brott, B.A. Pinsky, R.L. Erikson, Proceedings of the National Academy of Sciences of the United States of America 95 (1998) 963–968.
[3] S. Ota, S. Ishitani, N. Shimizu, K. Matsumoto, M. Ito, T. Ishitani, The EMBO Journal 31 (2012) 1904–1915.
[4] B. Ohkawara, K. Shirakabe, J. Hyodo-Miura, R. Matsuo, N. Ueno, K. Matsumoto, H. Shibuya, Genes & Development 18 (2004) 381–386.
[5] C.J. Thorpe, R.T. Moon, Development 131 (2004) 2899–2909.
[6] M. Kortenjann, M. Nehls, A.J. Smith, R. Carsetti, J. Schuler, G. Kohler, T. Boehm, European Journal of Immunology 31 (2001) 3580–3587.
[7] T. Ishitani, J. Ninomiya-Tsuji, K. Matsumoto, Molecular and Cellular Biology 23 (2003) 1379–1389.
[8] M.D. Meneghini, T. Ishitani, J.C. Carter, N. Hisamoto, J. Ninomiya-Tsuji, C.J. Thorpe, D.R. Hamill, K. Matsumoto, B. Bowerman, Nature 399 (1999) 793–797.
[9] C.E. Rocheleau, J. Yasuda, T.H. Shin, R. Lin, H. Sawa, H. Okano, J.R. Priess, R.J. Davis, C.C. Mello, Cell 97 (1999) 717–726.
[10] T. Ishitani, J. Ninomiya-Tsuji, S. Nagai, M. Nishita, M. Meneghini, N. Barker, M. Waterman, B. Bowerman, H. Clevers, H. Shibuya, K. Matsumoto, Nature 399 (1999) 798–802.
[11] M. Yamada, J. Ohnishi, B. Ohkawara, S. Iemura, K. Satoh, J. Hyodo-Miura, K. Kawachi, T. Natsume, H. Shibuya, The Journal of Biological Chemistry 281 (2006) 20749–20760.
[12] M. Herman, Development 128 (2001) 581–590.
[13] K.R. Siegfried, J. Kimble, J Development 129 (2002) 443–453.
[14] K.R. Siegfried, A.R. Kidd III, M.A. Chesney, J. Kimble, Genetics 166 (2004) 171–186.
[15] M.L. Mucenski, K. McLain, A.B. Kier, S.H. Swerdlow, C.M. Schereiner, T.A. Miller, D.W. Pietryga, W.J. Scott, S.S. Potter, Cell 65 (1991) 677–689.
[16] R.D. Allen III, T.P. Bender, G. Siu, Genes & Development 13 (1999) 1073–1078.
[17] C. Kanei-Ishii, J. Ninomiya-Tsuji, J. Tanikawa, T. Nomura, T. Ishitani, S. Kishida, K. Kokura, T. Kurahashi, E. Ichikawa-Iwata, Y. Kim, K. Matsumoto, S. Ishii, Genes & Development 18 (2004) 816–829.
[18] T. Kurahashi, T. Nomura, C. Kanei-Ishii, Y. Shinkai, S. Ishii, Molecular Biology of the Cell 16 (2005) 4705–4713.
[19] D.R. Calnan, A. Brunet, Oncogene 27 (2008) 2276–2288.
[20] S. Kim, Y. Kim, J. Lee, J. Chung, The Journal of Biological Chemistry 285 (2010) 8122–8129.
[21] A.A. Szypowska, H. de Ruiter, L.A. Meijer, L.M. Smits, B.M. Burgering, Antioxidants & Redox Signaling 14 (2011) 563–578.
[22] K. Satoh, J. Ohnishi, A. Sato, M. Takeyama, S. Iemura, T. Natsume, H. Shibuya, Molecular and Cellular Biology 27 (2007) 7623–7630.
[23] R. Ota, T. Kotani, M. Yamashita, Biochemistry 50 (2011) 5648–5659.
[24] T. Ishitani, T. Hirao, M. Suzuki, M. Isoda, S. Ishitani, K. Harigaya, M. Kitagawa, K. Matsumoto, M. Itoh, Nature Cell Biology 12 (2010) 278–285.
[25] F. Cichocki, M. Felices, V. McCullar, S.R. Presnell, A. Al-Attar, C.T. Lutz, J.S. Miller, Journal of Immunology (Baltimore, Md. : 1950) 187 (2011) 6171–6175.
[26] I. Takada, M. Mihara, M. Suzawa, F. Ohtake, S. Kobayashi, M. Igarashi, M.Y. Youn, K. Takeyama, T. Nakamura, Y. Mezaki, S. Takezawa, Y. Yogiashi, H. Kitagawa, G. Yamada, S. Takada, Y. Minami, H. Shibuya, K. Matsumoto, S. Kato, Nature Cell Biology 9 (2007) 1273–1285.
[27] L.R. Braid, W. Lee, A.C. Uetrecht, S. Swarup, G. Papaianni, A. Heiler, E.M. Verheyen, Developmental Biology 343 (2010) 178–189.
[28] N.H. Patel, E.E. Ball, C.S. Goodman, Nature 357 (1992) 339–342.
[29] Y.A. Zeng, M. Rahnama, S. Wang, W. Sosu-Sedzorme, E.M. Verheyen, Development 134 (2007) 2061–2071.
[30] K. Miyazono, Y. Kamiya, M. Morikawa, Journal of Biochemistry 147 (2010) 35–51.
[31] J. Jemc, I. Rebay, Biochemistry 76 (2007) 513–538.

[32] S.A. Morillo, L.R. Braid, E.M. Verheyen, I. Rebay, Developmental Biology 365 (2012) 267–276.
[33] J.C. Chiu, H.W. Ko, I. Edery, Cell 145 (2011) 357–370.
[34] K. Bae, I. Edery, Journal of Biochemistry 140 (2006) 609–617.
[35] S. Ishitani, K. Inaba, K. Matsumoto, T. Ishitani, Molecular Biology of the Cell 22 (2011) 266–277.
[36] T. Ishitani, S. Ishitani, K. Matsumoto, M. Itoh, Journal of Neurochemistry 111 (2009) 1104–1118.
[37] H. Takeshita, H. Sawa, Genes & Development 19 (2005) 1743–1748.
[38] I. Mirkovic, W.J. Gault, M. Rahnama, A. Jenny, K. Gaengel, D. Bessette, C.J. Gottardi, E.M. Verheyen, M. Mlodzik, Nature Structural & Molecular Biology 18 (2011) 665–672.
[39] T. Ishitani, S. Kishida, J. Hyodo-Miura, N. Ueno, J. Yasuda, M. Waterman, H. Shibuya, R.T. Moon, J. Ninomiya-Tsuji, K. Matsumoto, Molecular and Cellular Biology 23 (2003) 131–139.
[40] H. Kojima, T. Sasaki, T. Ishitani, S. Iemura, H. Zhao, S. Kaneko, H. Kunimoto, T. Natsume, K. Matsumoto, K. Nakajima, Proceedings of the National Academy of Sciences of the United States of America 102 (2005) 4524–4529.
[41] Z. Chen, T.B. Gibson, F. Robinson, L. Silvestro, G. Pearson, M.H. Cobb, Chemical Reviews 101 (2001) 2449–2476.
[42] Y. Miyata, E. Nishida, Biochemical and Biophysical Research Communications 266 (1999) 291–295.
[43] M. Krishna, H. Narang, Cellular and Molecular Life Sciences 65 (2008) 3525–3544.
[44] A.V. Khokhlatchev, B. Canagarajah, J. Wilsbacher, M. Robinson, M. Atkinson, E. Goldsmith, M.H. Cobb, Cell 93 (1998) 605–615.
[45] M. Adachi, M. Fukuda, E. Nishida, The EMBO Journal 18 (1999) 5347–5358.
[46] R. Philipova, M. Whitaker, Journal of Cell Science 118 (2005) 5767–5776.
[47] T.H. Shin, J. Yasuda, C.E. Rocheleau, R. Lin, M. Soto, Y. Bei, R.J. Davis, C.C. Mello, Molecular Cell 4 (1999) 275–280.
[48] E. Ohnishi, T. Goto, A. Sato, M.S. Kim, S.I. Iemura, T. Ishitani, T. Natsume, J. Ohnishi, H. Shibuya, Molecular and Cellular Biology 30 (2010) 675–683.
[49] J. Ji, T. Yamashita, A. Budhu, M. Forgues, H.L. Jia, C. Li, C. Deng, E. Wauthier, L.M. Reid, Q.H. Ye, L.X. Qin, W. Yang, H.Y. Wang, Z.Y. Tang, C.M. Croce, X.W. Wang, Hepatology 50 (2009) 472–480.
[50] S. Togi, O. Ikeda, S. Kamitani, M. Nakasuji, Y. Sekine, R. Muromoto, A. Nanbo, K. Oritani, T. Kawai, S. Akira, T. Matsuda, The Journal of Biological Chemistry 286 (2011) 19170–19177.
[51] A. Nifuji, H. Ideno, Y. Ohyama, R. Takanabe, R. Araki, M. Abe, M. Noda, H. Shibuya, Experimental Cell Research 316 (2010) 1127–1136.
[52] S. Zanotti, E. Canalis, Journal of cellular biochemistry 113 (2012) 449–456.
[53] E. Canalis, V. Deregowski, R.C. Pereira, E. Gazzerro, Journal of Endocrinological Investigation 28 (2005) 3–7.
[54] K.H. Jung, J.K. Kim, J.H. Noh, J.W. Eun, H.J. Bae, H.J. Xie, Y.M. Ahn, W.S. Park, L.Y. Lee, S.W. Nam, Journal of cellular biochemistry 110 (2010) 687–696.
[55] M. Peifer, P. Polakis, Science 287 (2000) 1606–1609.
[56] K.H. Emami, L.G. Brown, T.E. Pitts, X. Sun, R.L. Vessella, E. Corey, The Prostate 69 (2009) 1481–1492.
[57] G. Cui, Z. Li, B. Shao, L. Zhao, Y. Zhou, T. Lu, J. Wang, X. Shi, J. Wang, G. Zuo, W. Zhu, A. Shen, Journal of Clinical Neuroscience (2011) 271–275.
[58] H. Hikasa, J. Ezan, K. Itoh, X. Li, M.W. Klymkowsky, S.Y. Sokol, Developmental Cell 19 (2010).
[59] H. Hikasa, S.Y. Sokol, The Journal of Biological Chemistry 286 (2011) 12093–12100.
[60] R.G. Goold, P.R. Gordon-Weeks, J. Cell, Journal of Cell Science 114 (2001) 4273–4284.
[61] R.G. Goold, P.R. Gordon-Weeks, Molecular and Cellular Neurosciences 28 (2005) 524–534.
[62] F.Q. Zhou, W.D. Snider, Science 308 (2005) 211–214.
[63] X. Cai, M. Li, J. Vrana, M.D. Schaller, Molecular and Cellular Biology 26 (2006) 2857–2868.
[64] I. Dominguez, K. Itoh, S.Y. Sokol, Proceedings of the National Academy of Sciences of the United States of America 92 (1995) 8498–8502.
[65] X. He, J.P. Saint-Jeannet, J.R. Woodgett, H.E. Varmus, I.B. Dawid, Nature 374 (1995) 617–622.
[66] Y.H. Jin, H. Kim, M. Oh, H. Ki, K. Kim, Molecules and Cells 27 (2009) 15–19.
[67] L. Espinosa, J. Inglés-Esteve, C. Aguilera, A. Bigas, The Journal of Biological Chemistry 278 (2003) 32227–32235.
[68] E. Chalecka-Franaszek, D.M. Chuang, Proceedings of the National Academy of Sciences of the United States of America 96 (1999) 8745–8750.
[69] S.P. Davies, H. Reddy, M. Caivano, P. Cohen, The Biochemical Journal 351 (2000) 95–105.
[70] J.M. Beaulieu, T.D. Sotnikova, W.D. Yao, L. Kockeritz, J.R. Woodgett, R.R. Gainetdinov, M.G. Caron Proc, Proceedings of the National Academy of Sciences of the United States of America 101 (2004) 5099–5104.
[71] D. Burstein, P.J. Seeley, L.A. Greene, The Journal of cell biology 101 (1985) 862–870.
[72] P.S. Klein, D.A. Melton, Proceedings of the National Academy of Sciences of the United States of America 93 (1996) 8455–8459.
[73] C.M. Hedgepeth, L.J. Conrad, J. Zhang, H.C. Huang, L.M. Lee, P.S. Klein, Developmental Biology 185 (1997) 82–91.
[74] T.D. Gould, C.A. Zarate, H.K. Manji, The Journal of Clinical Psychiatry 65 (2004) 10–21.
[75] J.A. Quiroz, T.D. Gould, H.K. Manji, Molecular Interventions 4 (2004) 259–272.
[76] M.J. Berridge, C.P. Downes, M.R. Hanley, Cell 59 (1989) 411–419.
[77] J.M. Beaulieu, T.D. Sotnikova, W.D. Yao, L. Kockeritz, J.R. Woodgett, R.R. Gainetdinov, M.G. Caron, Proceedings of the National Academy of Sciences of the United States of America 101 (2004) 5099–5104.
[78] J.M. Beaulieu, S. Marion, R.M. Rodriguiz, I.O. Medvedev, T.D. Sotnikova, V. Ghisi, W.C. Wetsel, R.J. Lefkowitz, R.R. Gainetdinov, M.G. Caron, Cell 132 (2008) 125–136.