Double knock-out of Hmga1 and Hipk2 genes causes perinatal death associated to respiratory distress and thyroid abnormalities in mice

Raffaele Gerlini1,3, Elena Amendola1, Andrea Conte1,4, Valeria Valente1, Mara Tornincasa1,5, Sara Carmela Credendino1, Francesca Cammarota1, Chiara Gentile1, Luigi Di Guida1, Simona Paladino1, Gabriella De Vita1, Alfredo Fusco1,2 and Giovanna Maria Pierantoni1

Abstract
The serine–threonine kinase homeodomain-interacting protein kinase 2 (HIPK2) modulates important cellular functions during development, acting as a signal integrator of a wide variety of stress signals, and as a regulator of transcription factors and cofactors. We have previously demonstrated that HIPK2 binds and phosphorylates High-Mobility Group A1 (HMGA1), an architectural chromatinic protein ubiquitously expressed in embryonic tissues, decreasing its binding affinity to DNA. To better define the functional role of HIPK2 and HMGA1 interaction in vivo, we generated mice in which both genes are disrupted. About 50% of these Hmga1/Hipk2 double knock-out (DKO) mice die within 12 h of life (P1) for respiratory failure. The DKO mice present an altered lung morphology, likely owing to a drastic reduction in the expression of surfactant proteins, that are required for lung development. Consistently, we report that both HMGA1 and HIPK2 proteins positively regulate the transcriptional activity of the genes encoding the surfactant proteins. Moreover, these mice display an altered expression of thyroid differentiation markers, reasonably because of a drastic reduction in the expression of the thyroid-specific transcription factors PAX8 and FOXE1, which we demonstrate here to be positively regulated by HMGA1 and HIPK2. Therefore, these data indicate a critical role of HIPK2/HMGA1 cooperation in lung and thyroid development and function, suggesting the potential involvement of their impairment in the pathogenesis of human lung and thyroid diseases.

Introduction
Homeodomain-interacting protein kinase 2 (HIPK2) belongs to a family of nuclear serine–threonine kinases, originally identified as interactors of homeodomains-containing corepressor proteins1. Several studies have reported the crucial role of HIPK2 in many biological processes from apoptosis to cell proliferation2–5. Indeed, it is actively involved in cytokinesis, transcriptional regulation, signal transduction, and regulation of protein stability (reviewed in ref. 2–5). Generation of Hipk2 null mice (K2-KO) has been reported: they are significantly smaller than their wild-type littermates (WT) and show several neuronal defects6, including a reduction of midbrain dopamine neuron survival7 and apoptosis of cerebellar Purkinje cells, associated to several psychomotor behavioral abnormalities8.

The ability of HIPK2 to bind and phosphorylate several chromatin modifiers and transcription factors likely accounts for the wide spectrum of its biological functions. We have previously identified the nuclear non-histone chromatin high-mobility group A1 proteins as HIPK2 interactors and substrates5. HMGA1a and HMGA1b are encoded by the same gene (HMGA1) by alternative splicing. They belong to the HMGA protein family together with HMGA2, which is encoded by a different gene10,11. HMGA proteins are able to regulate the expression of several genes by binding to the minor groove of AT-rich DNA sequences.
and alter the chromatin structure through the interaction with several transcription factors. HMG proteins are abundantly expressed during embryogenesis and in most benign and malignant cancer tissues, whereas they are expressed at low levels in the normal adult tissues. HMG proteins have a well-established oncogenic activity, and usually their expression correlates with a highly malignant phenotype. HMG1 controls a wide spectrum of cell functions, including cell proliferation and survival, genomic stability, and autophagy. During development, HMG proteins are involved also in the regulation of body size, as demonstrated by the observation of the "super-pygmy" phenotype of the Hmga1/Hmga2 double knock-out (DKO) mice. Moreover, the biological activities of the HMG proteins are highly regulated by their post-translational modifications, such as acetylation, methylation, and phosphorylation, which have been associated with cellular transformation and proliferation.

Several studies evidence that HMG1 and HIPK2 share various fields of interactions, such as regulation of cell proliferation, apoptosis, and p53 activity. Indeed, whereas HMG1 is able to antagonize p53-driven transcription of apoptosis-related genes, HIPK2 instead is able to potentiate p53 pro-apoptotic activity by phosphorylating its Ser46. As evidence of their interaction, it has been finally catch-up with their WT counterpart (Fig. 2b, c and Supplementary Fig. 1).

To identify the reasons accounting for the cyanotic appearance and breathing dysfunction of the DKO mice which showed perinatal death, we performed a post mortem histological pulmonary examination. As shown in Fig. 2d, most of the lungs, in which Hmga1 and Hipk2 were absent, were characterized by the presence of collapsed, immature, sac-like alveoli with unexpanded alveolar space. This condition, known as neonatal atelectasis, is lined by immature cuboidal pneumocytes and characterized by abundant floccular eosinophilic material typical of an incorrect pulmonary development. Moreover, the observed atelectasis was often associated with prominent vasoconstriction of peribronchiolar arterioles (Fig. 2d) probably elicited by sustained hypoxia. As expected, no lung abnormalities were detected either in WT, A1-KO, and K2-KO mice (Supplementary Fig. 2A), or in the DKO mice which do not display respiratory distress (Supplementary Fig. 2b).

According to the criteria of mouse embryo development stages described by "Theiler" in "The House Mouse: Atlas of Mouse Development", the immature pulmonary pattern of newborn DKO mice is consistent with the earlier Theiler’s stages 25/26 of development, whereas WT mice show Theiler’s stage 27 at birth.

Therefore, the lack of both Hmga1 and Hipk2 results in high neonatal mortality, caused by the inability of the lungs to initiate or complete respiration after birth.

HMGA1 and HIPK2 proteins regulate the expression of surfactant proteins

The expression of genes encoding surfactant proteins (SP-A, SP-B, and SP-C) is limited to the pulmonary epithelial cells and is fundamental for their identity and maturation. The impairment of the regulation of such proteins results in respiratory failure and perinatal death, as shown in our study. The absence of HMGA1 and HIPK2, which are involved in the regulation of surfactant protein expression, causes a reduction in surfactant protein expression, leading to respiratory failure and perinatal death.
genes results in lethal neonatal respiratory distress in mice and humans (reviewed in ref. 26). During organogenesis, the earliest surfactant protein of the pre-type II cells is SP-C that, being expressed exclusively in the alveolar type II cells, is considered the most specific marker of appropriate alveolar maturation and successful remodeling of the lung (reviewed in ref. 27).

For these reasons, we checked the expression levels of surfactant genes in lungs of DKO mice, which showed perinatal death at P126, by quantitative PCR (qPCR). We found that \textit{Sp-A} and \textit{Sp-B} expression was almost undetectable in all the analyzed DKO mice, whereas \textit{Sp-C} expression was strongly decreased (~58%) in DKO mice with respect to their WT counterpart (Fig. 3a). Consistently, protein levels of surfactant protein C (SP-C) in lungs from DKO were lower than in lungs from WT mice at birth, as shown by western blot (Fig. 3b) and immunohistochemical (IHC) analyses (Fig. 3c and Supplemental Fig. S3). Noteworthy, surfactant proteins expression does not change in \textit{A1}-KO and \textit{K2}-KO mice (Supplementary Fig. 4), indicating that their expression decreases during embryonic development 29–31.

Because of the observed impairment of the surfactant protein expression in the lungs of DKO fetuses and newborns, we wondered whether HMGA1 and HIPK2 together are able to regulate the \textit{Sp-C} promoter. To this purpose, a luciferase assay activity was performed in HeLa cells, already used to study the regulation of \textit{Sp-C} promoter 32, in which an expression vector encoding the transactivator NKX2.1 (not endogenously expressed but required for the \textit{Sp-C} transcription) has been transfected together with HMGA1 and/or HIPK2-coding plasmids. As shown in Fig. 4c, both HMGA1 and HIPK2 significantly increased \textit{Sp-C} promoter activity in comparison with control-transfected cells. Consistently with a cooperative action of HMGA1 and HIPK2, the overexpression of both proteins resulted in a more drastic increase of \textit{Sp-C} promoter activity. Furthermore, to verify whether \textit{HMGA1} and \textit{HIPK2} silencing affects the expression of surfactant proteins also in vitro, we knocked down their expression with specific siRNA in the human bronchial epithelial cell line BEAS-2B. However, as shown in the Fig. 4d, there are no changes in the expression levels of surfactant protein after the interference of \textit{HMGA1} and/or \textit{HIPK2} in BEAS-2B cells. These results seem to indicate that the mechanisms regulating surfactant protein expression in vitro are different from those ones operating in vivo.
Since NKX2.1 (formerly called TTF-1 for Thyroid Transcription Factor-1), a member of the NKX2 family of homeodomain-containing transcription factors, is required for lung morphogenesis\textsuperscript{33} and the transcription of the surfactant genes\textsuperscript{32,34–36}, we evaluated whether the lung defects found in DKO mice were associated with an impairment of NKX2.1 expression. To this aim, we performed real-time qPCR and IHC analysis on lungs from WT and DKO mice at E17.5 and at P1 (Fig. 5a–d). We found that NKX2.1 level did not change either at E17.5 or at P1, suggesting that the strong reduction of surfactant expression in lungs of DKO mice was not dependent on a reduced Nkx2.1 expression. Furthermore, Nkx2.1 expression levels are not affected in A1-KO and K2-KO mice (Supplementary Fig. 5).

According to these results, the proper expression of surfactant genes requires in vivo the presence of both the HMG1 and HIPK2 proteins, and their lack mainly accounts for the atelectasis condition with a respiratory failure of DKO mice at birth.

**Impairment of thyroid in DKO mice**

The autopsic examination of thyroid glands of P1 DKO mice which showed the lung phenotype revealed also thyroid abnormalities. Indeed, thyroid follicles showed an irregular structure and were devoid of colloid, in contrast with the WT littermates (Fig. 6a, b). Then, we investigated the expression of the transcription factors which are required for the expression of the thyroid specific differentiation markers. In particular, we analyzed NKX2.1 by IHC (Fig. 6c, d), FOXE1 by IHC (Fig. 6e, f), and PAX8 by both IHC and qPCR (Fig. 6g, h, and k). FOXE1 and PAX8, but not NKX2.1, expression levels were strongly reduced in thyroids from DKO with respect to their WT counterpart. IHC and molecular analysis of late differentiation markers on DKO thyroid glands showed a reduction of expression levels of Thyroglobulin (TG) mRNA (Fig. 6k) and protein, which remained mainly localized at the periphery of the gland (Fig. 6i, j). The expression of other typical genes of differentiated thyroid follicular cells revealed that also Tireoperoxidase (Tpo) and Thyrotropin.
receptor (Tshr) mRNA was significantly reduced in DKO mice compared with the control mice (Fig. 6k). No abnormalities were detected in the morphology and in the IHC staining of NKX2.1, FOXE1, PAX8, and TG of single KO thyroids (Supplementary Fig. S6).

These data suggest that lack of both Hmga1 and Hipk2 genes impairs the expression of PAX8 and FOXE1 in thyroid gland and, consequently, of thyroid differentiation markers.

Discussion

The present study unravels an important role of HIPK2/HMGA1 functional interaction in determining neonatal survival, lung maturation, and thyroid development. Indeed, ~50% of HIPK2/HMGA1 DKO mice display perinatal lethality associated to respiratory distress and thyroid abnormalities. In particular, absence of both HIPK2 and HMGA1 expression resulted in impaired lung and thyroid development. In fact, the maturation of the lung alveolar epithelium is delayed in the DKO mice, as indicated by post-mortem analysis showing collapsed immature sac-like alveoli and atelectasis (Fig. 2d), then compromising a correct lung development. Noteworthy, both A1-KO and K2-KO mice were generated in a mixed genetic background C57BL6/Sv129J. Therefore, we retain that the heterogeneity of the DKO genotype may account for the different survival observed among litters and among pups of the same litter. It is likely that modifier genes affect the development of lung and thyroid pathology in DKO mice.

On the basis of the results reported here, it is likely that the impairment of the lung function is due to the ability of HIPK2 and HMGA1 to regulate the transcription of genes coding for surfactant proteins. In fact, surfactant factors are involved in the formation of a protein–lipid complex, which, by reducing alveolar surface tension, facilitates the processes that are impaired in DKO mice, such as alveolar space opening, lung fluid clearance, and inflation at birth37–39. Consistently with this hypothesis, we report that in the DKO mice which displayed the lung pathologic
phenotype, Sp-A and Sp-B expression was almost undetectable and Sp-C expression was strongly decreased (~58%) with respect to their WT counterpart (Fig. 3 and Supplemental S3), and that this is associated to the delayed maturation of pulmonary epithelial cells. Moreover, we demonstrate that the activity of the Sp-C promoter is regulated by both the HMGA1 and HIPK2 proteins, thus strongly suggesting that the drastic reduction of surfactant expression caused by the loss of HMGA1 and HIPK2 expression is responsible for the pathogenesis of respiratory distress. SP-A, B, C, and D proteins make the 10% of the complex and their expression is selectively confined to the respiratory epithelium of the lung. Interestingly, HMGA1 and HIPK2 in vitro silencing fails to reproduce the effects of Hmga1 and Hipk2 in vivo knock-out on surfactant proteins expression.
in the human bronchial epithelial cell line BEAS-2B. This discrepancy between in vitro and in vivo data is not surprising, and can have multiple explanations: (I) the effects of HMGA1 and/or HIPK2 depletion on surfactant expression might require the interaction with other cell types (non-cell-autonomous effects) and/or the presence specific components of the extra-cellular environment which are not present in the in vitro system; (II) the human model represents terminally differentiated cells, in which different mechanisms may regulate surfactant genes expression. Therefore, the in vitro model might not be suitable to reproduce the observations taken in vivo. According to this hypothesis, it has been already reported that in vitro data do not always reproduce in vivo observations41. The discrepancy between in vitro and in vivo data reported here points out the limitation of in vitro studies then underlining the need of studying the functional interaction of HMGA1 and HIPK2 in vivo.

It is established that disruption of the lipid complex, inability of surfactant relocation in the alveolar type II cell, and altered expression of SP-B and SP-C proteins could lead to severe respiratory distress in newborn infants and in mice42. These alterations compromise the function of the lung, which may, in turn, lead to lung atelectasis. It is noteworthy that mutations in the SFTPB human gene are associated with fatal respiratory distress in the neonatal period, and mutations in the SFTPC gene are more commonly associated with interstitial lung disease in older infants, children, and adults43. Moreover, it has been previously reported that HIPK2 regulates the hypoxia-inducible factor-1α (HIF-1α)44 that is responsible, among other transcription factors, for fetal lung development as it allows the fetus to quickly adapt to varying oxygen concentrations. Then, lung-specific HIF-1α KO leads to perinatal death in mice associated to impaired alveolar epithelial differentiation with consequent loss of surfactant protein expression45. Therefore, the loss of HIPK2 expression may affect surfactant levels also by modulating HIF-1α. However, the hypothesis that the loss of Hmga1 and Hipk2 could affect lung development by some other mechanisms, independent from their ability to activate surfactant protein expression, should be taken in consideration, and may be further investigated. In this perspective, it is known that mice carrying the impairment of both Ulk1 and Ulk2 genes show a pulmonary phenotype similar to the DKO mice, including perinatal respiratory distress and lethality, however in absence of a decreased surfactant expression46. The impairment of the...
Fig. 6 Thyroid morphology and differentiation in DKO newborn mice. a, b H&E staining of WT and DKO thyroid glands of mice at P1 (x 200 magnification). c–j NKX2.1, FOXE1, PAX8, and TG on WT c, e, g, i and DKO d, f, h, j thyroid sections (x 20 magnification). One representative experiment is shown. k RNA extracted from portion of neck containing thyroid gland of WT and DKO mice at P1 was analyzed by qRT-PCR for Pax8, Tg, Tpo, and TSHr expression. n = 3 WT mice and n = 6 DKO mice were analyzed. The Actin expression level has been used for normalization. Data are mean ± SD of a representative experiment performed in triplicate. * indicates significant difference $P < 0.05$, ***$P < 0.001$ (Student’s t test)
autophagic functions as a result of these mutations. The presence of autophagy is crucial for thyroid development, as previously described.

In summary, the results reported here indicate a new critical role of the HIPK2/HMGA1 functional interaction in lung and thyroid development and homeostasis. The involvement of possible alterations of both these genes might be investigated in diseases affecting lung and thyroid development.

Materials and methods

Generation of Hmga1−/−/Hipk2−/− mice, genotyping and primary MEFs

The Hmga1−/−/Hipk2−/− mice (DKO) were generated by crossing A1-KO and K2-KO mice. Embryonic day was estimated considering noon of the day of a vaginal plug as E0.5. Fetuses were collected at different embryonic stages (E) by cesarean section. Both A1-KO and K2-KO mice were generated in a mixed genetic background C57BL6/Sv129J and housed under diurnal lighting conditions (12 h dark/light). Experiments were performed according to the international guidelines for animal research. The experimental protocol was approved by the Animal Care Committee of the “Federico II” University of Naples. To assess the genotype of the mice, DNA was obtained from a small piece of tissue from the mouse. The tissue was incubated overnight at 60 °C with lysis buffer (50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, 1% SDS, 0.5 mg/ml proteinase K), and genomic DNA was extracted by adding 0.3 volumes of 6 M NaCl and precipitated with isopropyl alcohol. DNA concentration was determined using NanoDrop ND-1000 (NanoDrop, Wilmington, DE), and equal amount of DNA was used for PCR analysis. The annealing temperature for Hmga1 and Hipk2 alleles was 55 °C. The PCR products of Hmga1 and Hipk2 were separated on a 1% or 2% agarose gel, respectively. Gels were scanned with Chemidoc (Bio-Rad, Hercules, CA). The following primers were used:

- Hmga1-Fw 5′-AGAGACACAGATGGGAGACG-3′
- Hmga1wt-Rev 5′-TTTTACTAGGACCCTCATGG-3′
- Hmga1KO-Rev 5′-AAAGCGATCTGCTCAGACT-3′
- Hipk2-Fw 5′-TAGTACCAAGGGTCAAATGGAGT-3′
- Hipk2wt-Rev 5′-GCTCTCTCTAACCAAGCAGC-3′
- Hipk2KO-Rev 5′-CAAGGAGTTTGGAGCACCAGA-3′

For Hmga1 gene, the wt allele was amplified using Hmga1-Fw + Hmga1wt-Rev primers, and the knock-out allele was amplified using Hmga1-Fw + Hmga1KO-Rev primers. For Hipk2 gene, the wt allele was amplified using Hipk2-Fw + Hipk2wt-Rev primers, and the knock-out allele was amplified using Hipk2-Fw + Hipk2KO-Rev primers. MEFs have been isolated and genotyped as already described.

RNA extraction, RT-PCR, and quantitative RT-PCR

Total RNA from E17.5 embryos, cell lines, and tissues of newborn puppies was extracted as already described. cDNA was synthesized from total RNA through reverse transcription, and subsequent PCR amplification was performed as previously described. cDNA and qRT-PCR analysis for Ttf1, Pax8, Foxe1, Sp-C, Sp-A, Sp-B, Tg, Tpo, and Tshr was performed as already described. The following primers were used for qRT-PCR:

- mouse-Tg-Fw 5′-CATGGAATCTAATGCCCCAGAAC TG-3′
mice-Tg-Re 5′-TCCCTGTGACCTTTTGGAAATG-3′
mice-Tpo-Fw 5′-CAAGGGCTGGAGACCCCTATTGTCT-3′
mouse-Tpo-Re 5′-AACCTTTAGAGGTGCCTTGTCA-3′
mouse-Tshr-Fw 5′-TCCCTGAAAGACATCTTCCA-3′
mouse-Tshr-RE 5′-GATCCAGCTTTGTTCCATG-3′
mouse-Tff1-Fw 5′-TTACCGGACACATCGG-3′
mouse-Tff1-RE 5′-TGGCACTCATATTATCTGCGG-3′
mouse-Pax8-Fw 5′-CCATGGCTGTGAAGCAAGA-3′
mouse-Pax8-RE 5′-GCTTGGAGCCCTCATTACTAC-3′
mouse-Foxe1-Fw 5′-AAGCCGCCCCTACAGCTAC-3′
mouse-Foxe1-RE 5′-AACATTGCCTGGGCTGGG-3′
mouse-Sp-C-Fw 5′-GGTCTGATGGAGATCTCAC-3′
mouse-Sp-C-RE 5′-GATGAAGGGCTTGATG-3′
mouse-Sp-A-Fw 5′-CTGGAGAGACTGGAGGAC-3′
mouse-Sp-A-RE 5′-AAGCTCTCTACCAAGGTAGC-3′
mouse-Sp-B-Fw 5′-AACCCACACCTCTAGAAAC-3′
mouse-Sp-B-RE 5′-GTGACACCGTGCTATGG-3′
mouse-Actin-Fw 5′-CTAAGGCAAACGTGAAAG-3′
mouse-Actin-RE 5′-CATAGAGGCCATACAGGACA-3′
The primers used for Actin were above described.

The following primers were used for RT-PCR:
mouse-Hmgaid-Fw 5′-GCCAGAGCCAAAGACTG-3′
mouse-Hmgaid-RE 5′-GGAAGAACTGTGGATG-3′
mouse-Hipk2-Fw 5′-GACACAGGCTCAAGATTTG-3′
mouse-Hipk2-RE 5′-TCTGTCTCTGAAGTACGCTT-3′
mouse-G6pd-Fw 5′-GAAAGCAGGTGACCCCTTC-3′
mouse-G6pd-RE 5′-CATAGGAAATTCAGGGCAAAG-3′
The primers used for Actin were above described.

Protein extraction and western blotting

Protein extraction and western blotting experiments were performed as already described. The antibodies used were: anti-β-actin (sc-1616, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-γ-tubulin (sc-17787, Santa Cruz), anti-GAPDH (sc-32233, Santa Cruz), anti-vinculin (sc-7649, Santa Cruz), anti-PAX8 (ab18183, anti-NKX2.1,33, anti-FOXE1,64 anti-SP-B (ab3430, Millipore), anti-SP-C (90716, Abcam), anti-HIPK2 (Novus Biologicals), anti-HMGAI65. Densitometric analyses of western blot were performed with Image J software.

Histology and immunohistochemistry

When not spontaneously dead, animals were killed by decapitation. Sections of neck containing thyroid gland and lungs were fixed overnight at 4 °C in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, dehydrated through ethanol series, cleared in xylene and embedded in paraffin. For histological analysis, 7 mm sections were stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO), according to the manufacturer’s instructions. For immunohistochemical analysis, 7 mm sections were dewaxed by standard techniques. Heat treatment was performed for antigen retrieval in sodium citrate buffer, pH 6, followed by permeabilization by incubating sections in PBS containing 0.2% Triton X-100. Incubation with primary antibodies was performed overnight at 4 °C in blocking buffer (3% BSA, 5% goat serum, 20 mM MgCl2, 0.3% Tween 20 in PBS). Endogenous peroxidase activity was quenched with 35% H2O2 in methanol at room temperature, and chromogenic reactions were carried out according to the Vectastain ABC kit protocol (Vector Laboratories, Burlingame, CA). No nickel was added in the chromogenic reaction for supplementary immunohistochemistry data. The following primary antibodies were used: anti-proSP-C (90716, Abcam); anti-SP-A (ab3420, Millipore); anti-SP-B (ab3430, Millipore); anti-PAX8,62 anti-NKX2.1,33 anti-FOXE1,63 anti-TG rabbit polyclonal antibody (Dako). Biotinylated anti-rabbit IgG 1:200 was used for detection of primary antibodies (Vector Laboratories, Burlingame, CA).

Cell cultures, transfections, and luciferase assays

HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Euroclone) supplemented with 10% fetal bovine serum (Invitrogen). The pcDNA3.1-Hmgai1b and pCEFL-HA-Hipk2 vectors were previously described.65,66 Cells were transfected as previously described.67 In brief, HeLa cells were plated in six-well plates and after 24 h were transfected with 200 ng of pLuc-SP-C34 with 50 ng of pCMV-Renilla plasmid and with pCMV-TTF-1(Nkx2.1) expression vector35 alone or with different amount of pcDNA3.1-Hmgai1b and pCEFL-HA-Hipk2 expression vectors.

BEAS-2B cells were grown in LHC-9 serum-free medium (Gibco, Invitrogen) supplemented with l-glutamine, retinoic acid, epinephrine, gentamicin, insulin, hydrocortisone, EGF, transferrin, bovin pituitary extract, and T3 supplemented with RNAi was performed transfecting siRNA [HMGAI:Stealth siRNAs (Set of 3)] HSS142459, HSS142460, HSS142461, Cat. #1299001, (Life Technologies-Invitrogen); HIPK2: Stealth siRNAs (Set of 3) HSS120797, HSS120796, HSS178947, Cat. #5258224. (Life Technologies-Invitrogen)] using Lipofectamine RNAI MAX (Thermofisher), according to manufacturer’s instructions.

Statistical analysis

Data were analyzed using a two-sided unpaired Student’s t test (GraphPad Prism, GraphPad Software, Inc.). Values of P < 0.05 were considered statistically significant. All animal experiments included at least three biological replicates. Data are expressed as mean ± SD.
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Author details
1Department of Molecular Medicine and Medical Biotechnology, University of Naples, Naples, Italy. 2Institute of Endocrinology and Experimental Oncology, CNR, Naples, Italy. 3Present address: Institute of Experimental Genetics, Helmholtz Zentrum, Munich, Germany. 4Present address: Lymphocyte Nuclear Biology, NIAMS, NIH, Bethesda, MD, USA. 5Present address: AXXAM S.p.a.-OpenZone, Bresso, Milan, Italy

Authors’ contributions
R.G. and F.C. performed the mouse genetics and quantitative PCR experiments; A.C., C.G., V.V. and M.T. performed the cellular and molecular experiments; E.A., S.C. and S.P. performed immunohistochemical experiments; L.D.G. contributed to the mouse handling and genetics; G.D.V. contributed to critical review of the manuscript; R.P. and A.F. coordinated the work and wrote the manuscript. All the authors discussed the results and revised the manuscript text.

Conflict of interest
The authors declare that they have no conflict of interest.

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