We are IntechOpen, the world’s leading publisher of Open Access books
Built by scientists, for scientists

3,900 Open access books available
116,000 International authors and editors
120M Downloads

154 Countries delivered to
TOP 1% Our authors are among the most cited scientists
12.2% Contributors from top 500 universities

WEB OF SCIENCE™
Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com
The Mitotic Protein Kinase Haspin and Its Inhibitors

Omid Feizbakhsh, Matthieu Place, Xavier Fant, Frédéric Buron, Sylvain Routier and Sandrine Ruchaud

Abstract

Haspin is an atypical serine/threonine protein kinase essential to mitosis. Unlike other protein kinases, its kinase domain does not require phosphorylation in order to be activated and bears very high substrate specificity and selectivity. Few substrates have been identified so far. Haspin phosphorylation on threonine 3 of Histone H3 from prophase to anaphase participates to centromeric Aurora B localization and ensures proper kinetochore-microtubule attachment. Haspin is also involved in the maintenance of centromeric cohesion and the mitotic spindle. Inhibitors have been developed and provided tools to dissect Haspin function. The kinase is now considered as a potential therapeutic target against cancer. We discuss here the latest findings on this essential mitotic protein.

Keywords: Haspin kinase, mitosis, inhibitors, H3T3ph, centromere

1. Introduction

Protein kinases play an important role in cell cycle regulation. Together with protein phosphatases, they regulate the phosphorylation status of thousands of substrates, including proteins ensuring cell cycle progression. Cell division, that is, mitosis, is a crucial step of the cell cycle and is essential to genomic stability. Mistakes during this process can cause various developmental diseases and cancers. Its orchestration is highly regulated by various families of protein kinases, including cyclin-dependent kinases (Cdk), Aurora kinases, polo-like kinases (Plks), and NimA-related kinases (Neks) whose roles in mitosis are well documented [1, 2]. Haspin is a serine/threonine kinase discovered in the early 1990s essential to mitosis. Despite recent progress, regulation of its activity and its biological functions is still poorly understood. Haspin is involved in chromosome alignment, centromeric cohesion, and spindle stability making it a potential target against cancer. Latest data from the literature concerning this protein kinase as well as pharmacological inhibitors are presented.
2. Discovery

Haspin mRNA was first detected in mouse germ cells in 1994 and named germ cell–specific gene 2 (GSG2) [3]. The coded protein localizes in the nucleus of germ cells and showed kinase activity. It was subsequently renamed haploid germ cell–specific nuclear protein kinase (Haspin) [4]. Haspin mRNA is found in diploid cells of many proliferative tissues such as testis, thymus, bone marrow, and spleen as well as in many proliferative cell lines [5–7]. Its expression is comparatively reduced in somatic tissues. In addition, Haspin orthologs were found in several eukaryotes, such as yeasts, plants, flies, fishes and mammals, and a large group in Caenorhabditis elegans. Phylogenetic analysis indicates that Haspin proteins form a new family of eukaryotic proteins kinases (ePK) [5].

3. Haspin, an atypical kinase structure

Human Haspin is a 798 amino acid serine/threonine protein kinase. The N-terminal part (aa 1–469) is the less conserved among species and thought to act as a regulatory domain. The well-conserved C-terminal part (aa 470–798) corresponds to the catalytic kinase domain [8–10]. To date, Haspin kinase domain was crystallized in the presence of ATP analogs such as 5-iodotubercidin (5-ITu) or in the presence of a specific substrate of the kinase, Histone H3 [8, 10, 11]. Disorganization of the N-terminal domain prevented, so far, crystallization of the entire protein.

The structure of human Haspin kinase domain (aa 470–798) shows similarity to the kinase domain from other kinases of the ePK family. As most protein kinases, it includes a small lobe on the N-terminal side and a large lobe on the C-terminal side. A substrate-binding site and an ATP binding pocket are found between the two lobes. The catalytic domain of Haspin displays specific structural features that are not observed in other members of the ePK family.

Compared to canonical protein kinases, Haspin structure revealed several unique and specific structural features that are highly conserved in several species. These characteristics result from amino acid insertion, deletion, or changes in the protein sequence of its catalytic domain in comparison to other ePKs [8, 10]. The structure of most protein kinases is generally very dynamic, allowing a kinase to transform, by a conformational change, from an inactive to an active state through phosphorylation or interaction with a partner [12]. On the contrary, Haspin kinase domain is rigid and fixed in a constitutively active conformational state [8, 10, 11] (Figure 1A).

This stability is achieved by the combination of different structural elements. Mainly, the generally mobile glycine rich P-loop is stabilized in Haspin by the insertion of an additional helix in the upper lobe called either upper lobe helix (ulfH) [8] or αC’ helix [10]. This helix insertion is mostly conserved throughout Haspin orthologs apart from the fission yeast Alk1 and Alk2. Usually, mobile αC helix in the small N-terminal lobe is also stabilized by a number of hydrophobic contacts. Haspin activation segment is another atypical structural element. In most kinases, the activation segment has a regulatory purpose, acquiring an active...
conformation upon phosphorylation and allowing substrate binding. Haspin activation segment is stabilized in a constitutively active conformation [8, 10].

Finally, Haspin bears a very specific substrate-binding site. Knowing that histone H3 tail (positively charged) is a specific Haspin substrate suggested a negatively charged binding site as depicted on Figure 1B. Maiolica et al. provided insights into this peculiar substrate-binding site resolving the crystal structure of Haspin kinase domain bond to the first seven residues of Histone H3 [11]. The study revealed that three residues of the latter, Ala1, Arg2, and the phospo-acceptor site Thr3, are deeply anchored in the substrate hydrophilic binding site of Haspin. These peculiarities create a highly selective substrate-binding site [11].

4. Haspin substrates

Very few substrates of Haspin have been identified and characterized so far. Histone H3 was the first Haspin substrate to be identified. It is specifically phosphorylated on Thr3 [8, 10, 13].

Figure 1. Haspin kinase domain 3D structure. (A) Insertion elements, αC helix, β9 and β9′, and β hairpin are indicated. (B) Representation of Haspin surface electrostatic charges; the negatively charged substrate-binding site is circled.
This phosphorylation (H3T3ph) was demonstrated both in vitro and in several cell lines by immunofluorescence, using histone H3 Thr3 phospho-specific antibodies (Figure 5 upper panel). Haspin depletion by siRNA eliminates H3T3 phosphorylation in mitotic cells, and ectopic overexpression of Haspin leads to abnormal H3T3 phosphorylation levels in interphase cells confirming that H3T3ph is specific of Haspin activity [7, 14, 15]. H3 is phosphorylated on Thr3 through most of mitosis. Kurihara et al. showed that Arabidopsis thaliana Haspin, AtHAspin, is an H3T3ph kinase. They have further demonstrated that AtHaspin phosphorylates both Thr3 and Thr11 of Histone H3 in vitro [16, 17]. It is to be noted that Haspin-homologous proteins in budding yeast, Alk1 and Alk2, have not shown any ability to phosphorylate histones [18], whereas the fission yeast Haspin-related kinase, Hrk1, has been shown to be the major H3T3 phosphorylating kinase in this species [19].

Histone macroH2A is an histone variant found enriched on inactive X chromosome of female mammals [20]. Several studies demonstrated that histone macroH2A functions both as a positive and a negative regulator of gene transcription. A phosphoproteomic study showed that inhibition of Haspin by 5-Tiu led to a sharp decrease in serine phosphorylation of histone macroH2A [11]. This phosphorylation was confirmed in vitro and in HEK293 cells, where overexpression of Haspin caused hyper-phosphorylation of Histone macroH2A on Ser137, the latter being inhibited by 5-Tiu. It has also been reported that the macro domain of Histone macroH2A controls the levels of Ser10 and Thr3 phosphorylation of histone H3 in human cells and would be involved in controlling chromatin condensation [21, 22]. The functionality of Haspin in these mechanisms remains to be confirmed.

CENP-T is a component of the constitutive centromere-associated network (CCAN), which plays a central role in kinetochore assembly, mitotic progression, and segregation of chromosomes [23]. CENP-T has been identified as substrate of Haspin by consensus site prediction (see below), and its phosphorylation on several sites confirmed by in vitro kinase assay [11].

The Haspin kinase substrate recognition motif has been determined by positional scanning-oriented peptide library screening (PS-OPLS) as A/V-R-T/S-K-(X-no D/E) with a preference for threonine residues [11]. Acidic residues have been shown to impair Haspin recognition when in the surrounding of the phosphorylation site [11].

5. Haspin biological function

5.1. Haspin localization

Haspin is constitutively expressed throughout the cell cycle, unlike other mitotic kinases such as Aurora B and Plk1, which are degraded at the end of mitosis [13, 24, 25]. So far, the precise cellular localization of the endogenous protein could not be determined due to lack of immunofluorescence-specific antibodies to Haspin. However, several overexpression studies have reported localization of GFP- or Myc-tagged Haspin in different eukaryotic cell lines (HeLa, U-2 Os, Hek293, COS-7) using time-lapse video microscopy or immunofluorescence staining techniques or time-lapse video microscopy [4, 13]. All these studies showed that Haspin localizes in discrete foci and nucleoli in the nucleus during interphase. It is to be noted
that the N-terminal domain of human Haspin exhibits two potential nuclear localization signals (NLSs) that are conserved in mice and rats [9]. Localization pattern in mitosis is more complex (Figure 2).

At the end of G2/onset of prophase, Haspin appears associated with condensed chromosomes until anaphase B. Myc:Haspin is observed along chromosome arms with a clear concentration at centromeres [13]. GFP:Haspin was also detected at the centrosomes and mitotic spindle in prometaphase cells until telophase, where a weak signal is detected in the midbody [13]. Nuclear and chromosomal localization throughout mitosis of endogenous Haspin:YFP knocked-in have been recently confirmed by video microscopy [26]. Figure 2 shows the location of Haspin during the various stages of the cell cycle with emphasis on mitosis (Figure 2). Phosphorylation of histone H3 on Thr3 was also examined in plants. In most of the species studied, it appears on chromosomes at the end of G2 phase and disappears during anaphase [27], in contrast to mammals, phosphorylation of Thr3 is seen primarily at pericentromeres in prophase and then along chromosome arms during prometaphase [27, 28].

5.2. Specific localization of Haspin during meiosis

GFP:Haspin co-localizes with chromatin and H3T3ph during all meiotic stages [29, 30]. Notably, H3T3ph levels were increased with overexpression of exogenous Haspin, thus confirming that Haspin phosphophorylates H3T3 in oocytes. During metaphase I, Haspin is detected at the centromeres and along sister chromatids. After metaphase I, GFP:Haspin is also located in a discrete region of the oocyte’s cortex which is in the immediate vicinity of chromatin and the spindle [29]. Haspin was also weakly detected as filamentous aggregates on the spindle.

![Figure 2](http://dx.doi.org/10.5772/intechopen.70732)
At anaphase I/telophase I transition, Haspin is translocated from chromosomes to midbody [29, 30].

5.3. How is Haspin recruited onto chromosomes?

A yeast two-hybrid screen of cohesin-related proteins on the fission yeast Haspin homolog Hrk1 identified an interaction with the cohesin-associated protein Pds5 [19]. The interaction was confirmed in cells using a model in which Pds5:mCherry:LacI was tethered onto a specific location on a chromosome arm through a LacO/LacI system onto which Hrk1:GFP was shown to co-localize (Figure 3) [19].

Vertebrates have two version of Pds5 protein, Pds5A and B [31, 32]. Carretero et al. demonstrated that Pds5B-deficient MEF cells showed a decreased activation of Aurora B and Haspin at centromeres and an impaired centromeric localization of Aurora B suggesting that Pds5B may be involved in the recruitment of Haspin on centromeres [33]. The recruitment of Haspin by Pds5B has been confirmed in human cell lines by Hindriksen et al. [26]. Using a LacO/LacI system in which Pds5B:RFP:LacI was shown to recruit Haspin:YFP on a LacO repeats inserted on chromosome 1 of human U-2 OS cells [26].

Two recent studies in Xenopus and yeast showed that SUMOylated DNA topoisomerase IIα C-terminal domain can bind Haspin and regulate its localization at centromeres [34, 35] (Figure 3).

5.4. Haspin function in mitosis

In vertebrate cell lines, depletion of Haspin by siRNA or treatment with specific inhibitors leads to a substantial decrease in histone H3 Thr5 phosphorylation during mitosis. Moreover,
cells display many remarkable defects in mitosis. Prometaphase and metaphase duration are increased due to severe chromosome alignment defects [6, 7, 13] (Figure 4).

Mitosis duration, measured by video microscopy, is increased up to several hours in Haspin-depleted U-2 OS cells [36]. Mitotic spindles are disorganized with often extra centrosome-like foci. Impaired centromeric cohesion and premature separation of chromatid have been reported in Higgins et al. [36].

Haspin has been shown to be the major H3T3 phosphorylating kinase in various organisms [7, 8, 10, 11, 13, 15]. Phosphorylation on H3T3 appears first at the end of G2 phase of the cell cycle and disappears during anaphase B (Figure 5 upper panel). H3T3ph is well marked in prophase. At this stage, it is nuclear and more precisely located on condensing chromosome arms. During prometaphase, phosphorylation is concentrated on centromeres in a region delimited by centromeric CENP-A (inner centromere). The phosphorylation decreases rapidly at anaphase and can still be observed on telomeres present in the vicinity of Aurora B activity area on the midzone. It is no longer detected on chromosomes, when cells are in late telophase (Figure 5 upper panel) [6, 7, 13, 14].

The H3T3ph-dephosphorylating enzyme has been shown to be the PP1γ phosphatase specifically targeted to anaphase chromosomes by its regulatory subunit Repo-Man [37, 38]. Further studies showed that Repo-Man targeting to chromosomes is negatively regulated through Aurora B phosphorylation explaining the persistent H3T3ph signal observed on telomeres at anaphase [37].

Histone H3 phosphorylated on threonine 3 is directly recognized by the conserved BIR domain of Survivin, a member of the chromosomal passenger complex (CPC) [14, 19, 39], thus

![Figure 4. Haspin depletion by siRNA. Immunofluorescence images of Haspin and control siRNA on U2 OS cells. Haspin-depleted cells show chromosome alignment defects, impaired spindles, and ectopic spindle poles.](image-url)
anchoring the CPC at centromeres. The CPC is a complex of four subunits, Survivin, Borealin, INCENP, and the Aurora B kinase. Aurora B is an essential kinase, which regulates mitotic progression, including spindle assembly checkpoint, condensation, and chromosomal bi-orientation and cytokinesis [40–42]. Additionally, Aurora B phosphorylates Haspin N-terminus

Figure 5. Haspin activity on Thr3 of histone H3. Upper panel, localization of Haspin activity on Thr3 of histone H3 along the cell cycle in mammal cells. Lower panel, schematic representation of Haspin-aurora B positive feedback loop at centromere.
on several sites allowing its over-activation and creating a positive feedback loop triggering the accumulation of CPC at the centromeres [14] (Figure 4 lower panel).

The recruitment of CPC at centromere is not only dependent on H3T3ph by Haspin. A second parallel pathway involving histone H2A phosphorylation on Thr120 by Bub1 creates a binding site for Shugoshin, a protein involved in the protection of centromeric cohesion. Shugoshin binds directly to Survivin in yeast and to Borealin CPC member in human in a comparable manner [14, 43, 44].

Therefore, one of the major functions of Haspin is, together with Bub1 kinase, to bring the CPC at centromeres (Figure 6).

Haspin has been shown to be involved in chromosomal cohesion. Defects in chromosome alignment in Haspin-depleted cells are probably due, at least in part, to a premature loss of sister chromatid cohesion [6].

A recent study has demonstrated that, during mitosis, Haspin binds to the cohesin-associated protein Pds5B [45]. During prophase and prometaphase, sister chromatids resolution occurs through cohesion release upon binding of Wapl protein to Pds5B. Zhou et al. showed that Haspin interaction with Pds5B inhibits Wapl binding, protecting from premature centromeric cohesion loss [45].

5.5. Regulation of Haspin activity

The N-terminal domain of Haspin appears to be involved in both the intracellular localization of the protein and in the regulation of its kinase domain activity. Indeed, it has been shown that presence of the N-terminal domain changes the phosphorylation kinetics of Histone H3 substrate peptides, when compared to the catalytic C-terminal part alone, increasing the $K_m$ for ATP and lowering the affinity for Histone H3 [10]. Thus, the N-terminal domain has the potential to modulate the activity of the enzyme [10].

Figure 6. Haspin, together with Bub1, is required for anchoring the CPC at centromere. Svn: Survivin; INC: INCENP; Bor: Borealin; Aur B: Aurora B; Sgo: Shugoshin; CPC: Chromosomal passenger complex.
Haspin is expressed throughout the cell cycle [13, 46]. However, it is highly phosphorylated during mitosis [13]. Phosphoproteomic studies showed that these phosphorylations are on the N-terminal domain of the protein, where phosphorylation consensus sites for Cdk1, Plk1, and Aurora B are present [45–47]. Phosphorylation events on the N-terminal domain at the onset of mitosis trigger conformational changes and influence Haspin kinetics parameters (see above). Haspin phosphorylation by Cdk1/cyclin B starts on T128 of human Haspin (T206 in *Xenopus laevis*) [47, 48]. Gheniou *et al.* showed that Xenopus Haspin auto-inhibits itself during interphase through a conserved basic site in its N-terminus part close to its kinase domain [47]. This auto-inhibition is released through Cdk1 phosphorylation of Haspin N-terminus followed by the recruitment of Plk1 on the Cdk1 phospho-site and its activation. Activated Plk1 phosphorylates several sites on Haspin N-terminus releasing its activity in a timely manner at the beginning of mitosis triggering H3T3 phosphorylation and CPC recruitment at centromeres [47, 48]. Furthermore, Wang *et al.* showed that Aurora B further phosphorylates Haspin N-terminus enhancing its ability to generate H3T3 phospho-sites for Survivin/CPC binding [49] (Figure 5 lower panel). Another recent study showed that Aurora A also phosphorylates Haspin N-terminus triggering the Aurora B/Haspin feedback loop [50].

Several reports showed that H3T3 phosphorylation by Haspin is regulated by modifications on adjacent residues Arg2 and Lys4. As such, methylation on Arg2 as well as acetylation and methylation on Lys4 strongly decreased the ability of Haspin to phosphorylate Thr3 [8, 51]. These results imply a likely epigenetic regulation of Haspin and Aurora B activities.

5.6. Haspin function in meiosis

Studies of Haspin function during meiosis were performed on mouse oocytes using small molecule inhibitors and overexpression. Similarly to mitosis, Haspin phosphorylates Thr3 of histone H3 [29, 46]. This phosphorylation is necessary for accurate meiosis including chromatin condensation and formation of the microtubule assembly checkpoint, ensuring faithful segregation of chromosomes during meiosis I [29, 46]. Furthermore, Haspin phosphorylation on H3T3 has been shown to be required for Aurora C kinase proper localization during meiosis [29, 46]. During meiosis I, in contrast to mitosis, where Haspin is involved in CPC targeting to centromeres, Haspin regulates Aurora C localization to the inter-chromatid axis [29, 30]. Inhibition of Haspin by 5-ITu showed a failure to organize microtubules and an increase in microtubule organizing centers (MTOCs) as well as an impaired localization of Aurora C at this location. These results suggested a new role for Haspin in the regulation of MTOCs clustering during meiosis and Aurora C localization at MTOC supporting the idea of different functions of Haspin in meiosis compared to mitosis [30].

6. Haspin as a therapeutic target

Mitotic protein kinases are considered as targets of choice for drugs developed by the pharmaceutical industry [52]. Because of its role in controlling the activity of Aurora B and in maintaining the cohesion of centromeres and spindle poles, Haspin has become a relevant target for cancer therapy and is considered as an emerging anti-mitotic drug target [53, 54].
The fact that Haspin is an atypical ePK with a divergent structure may lead to the development of inhibitors with fewer side effects [4, 5]. Haspin inhibitor CHR6494 described by Huertas et al. showed antitumor activity in a xenograph mouse model [53]. Haspin is also overexpressed in some malignant tumors such as Burkitt’s lymphoma and chronic lymphocytic leukemias [55, 56]. In addition, Haspin was identified in a whole kinome siRNA screen, together with Plk1, as one of the top hit kinases, whose depletion decreased both cell viability and estrogen receptor transcriptional activity in MCF7 breast cancer cells [57]. Thus, Haspin may represent a new anti-cancer therapeutic target.

7. Haspin inhibitors

There are only few reports on conception of Haspin inhibitors (Figure 7). Most publications reported the evaluation of molecules on a kinase panel, including Haspin kinase.

One of the first molecules used in researches on Haspin was the well-known 5-ITu for its potent inhibition of adenosine kinase. This nucleotide-like molecule inhibits strongly Haspin with IC$_{50}$ ranged between 5 and 9 nM. Initially, 5-ITu was mostly used to get a better understanding of Haspin structure and could also be considered as a tool for biological studies. 5-ITu was recently used to assess biological function of Haspin on the cell cycle, especially during mitosis and meiosis (see above). Cuny et al. described the screening, synthesis, and biological functions of Haspin inhibitors described in the literature.
evaluation of two compounds with interesting activities on Haspin [58]. The study was mostly devoted to target Dyrk2 and Haspin kinases for their role on proliferating cells. After a screening of 140,000 species, an acridine analog demonstrated an interesting profile, and authors isolated LDN-192960, which showed remarkable inhibition of Haspin kinase (IC\textsubscript{50} = 10 nM). In 2012, the same team synthesized a library of harmine derivative with the same amino-alkyl chain. The newly generated LDN-211898 is described as an inhibitor active at submicromolar concentration against Haspin in an \textit{in vitro} assay (100 nM). Recently, Novartis realized a large screen using Melk inhibitors and found that the compound Melk8a had the best inhibition potency against Haspin (IC\textsubscript{50} = 190 nM). However, this compound also showed activities below 1 \textmu M of other kinases including Gsk3, Cdk2, Akt1, Flt3 and was therefore not selective. This year, Pastor Fernández \textit{et al.} described the synthesis of tricyclic compounds as new kinases inhibitors [59]. Mostly, the patented molecules have strong activities against Cdk8, Cdk19, and Haspin, as shown for inhibitor L1 (IC\textsubscript{50} = 9 nM). A similar approach was used by Chen \textit{et al.}, who first described SGI-1776 as a Pim1 inhibitor [60]. \textit{In vitro} evaluation of this compound on a panel of kinases gave an IC\textsubscript{50} of 34 nM on Haspin. This study was the starting point for screening of other imidazoipyridazine as strong Haspin inhibitors. In 2012, Huertas \textit{et al.} described \textit{in vitro}, \textit{in cells}, and \textit{in vivo} activities of a little imidazoipyridazine named CHR6494 [53]. This molecule seems to be an ATP competitive drug commonly denominated as a type I kinase inhibitor showing a strong inhibition of Haspin with a remarkable IC\textsubscript{50} of 2 nM.

Kestav \textit{et al.} developed another original type of inhibitors. They synthetized conjugates bearing an aromatic fragment fused to a peptide mimicking the N-terminus of histone H3. Their best compounds showed a K\textsubscript{d} of 0.42 nM on Haspin kinase with a good selectivity index [61].

8. Conclusion

Haspin protein kinase was discovered two decades ago. Despite several years of research, the only well characterized substrate, with a specific function, is Histone H3 Thr3. This atypical kinase and its essential role in the regulation of CPC activity in space and time along mitosis, through Thr3 phosphorylation of Histone H3, has become a very attractive subject. The latest findings reviewed here show that there is still much to discover about the function and regulation of this kinase. Although Haspin inhibitors have shown to be very useful tools in dissecting the kinase function in diverse biological mechanisms within multiple organisms, we expect to see their development toward therapeutic drugs in the coming years.

Acknowledgements

We thank Dr. Jonathan Dorival (Roscoff Biological Station, UMR8227) for his help on illustrating Figure 1 and Dr. Julien Duez (Roscoff Biological Station, USR3151) for helpful criticisms on text and figures. Our work is supported by La Ligue Contre le Cancer, Fondation ARC, Cancéropôle GrandOuest and GIS Biogenouest. OF was supported by a Brittany Region ARED Doctoral fellowship.
Author details

Omid Feizbakhsh1, Matthieu Place2, Xavier Fant1, Frédéric Buron2, Sylvain Routier2 and Sandrine Ruchaud1*

*Address all correspondence to: sruchaud@sb-roscoff.fr

1 Protein Phosphorylation & Human Diseases, Station Biologique de Roscoff, Sorbonne Universités, Roscoff, France
2 Institut de Chimie Organique et Analytique, Université d’Orléans, Orléans, France

References


