Phosphatase of regenerating liver-3 (PTP4A3/PRL-3) is a dual-specificity phosphatase that is upregulated in various types of cancers and is related to poor prognosis and aggressive tumor behavior. The expression level of PRL-3 is elevated in response to several anti-apoptotic cytokines, including IL6, in cancer cells from patients with multiple myeloma (MM) and can promote survival and migration. Here, it is demonstrated that PRL-3 activates Src kinase in the IL6-dependent MM cell line INA-6. Inhibition of PRL-3 by a small-molecule inhibitor of PRL-3 or by shRNA resulted in inactivation of Src. In addition to activation of Src, PRL-3 also activated the Src family kinase (SFK) members LYN and HCK in INA-6 cells. Forced expression of catalytically inactive mutant PRL-3 decreased the activation of these three SFK members while the total level of HCK and FYN remained elevated. Inhibitors of Src increased sensitivity of cells overexpressing PRL-3 to the PRL-3 inhibitor through joint downregulation of both PRL-3 and Mcl-1. In conclusion, PRL-3 protected MM cells against apoptosis by dysregulating both the total levels and the activation levels of specific SFK members that are important for IL6 signal transduction in MM cells. Eventually, this led to increased levels of Mcl-1.

Implications: This study suggests PRL-3 and SFKs are key mediators of the IL6-driven signaling events and points to both PRL-3 and SFK members as potential targets for treatment of MM.

The content is from "Src Family Kinases Are Regulated in Multiple Myeloma Cells by Phosphatase of Regenerating Liver-3" by Pegah Abdollahi, Esten N. Vandsemb, Magnus A. Hjort, Kristine Misund, Toril Holien, Anne-Marit Sponaas, Torstein B. Rø, Tobias S. Slørdahl, and Magne Berset.

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Our group has previously shown that PRL-3 is a downstream target of IL6 in MM by demonstrating that PRL-3 mRNA and protein is upregulated in response to IL6. We also showed that PRL-3 is involved in migration of MM cells and that it increases the level of the antiapoptotic Bcl-2 family member Mcl-1 (14, 22). In this study, we wanted to explore closer the signaling pathways regulated by this phosphatase in order to better understand its oncogenic properties. Several SFK members are involved in IL6-induced signaling (23, 24), and others have reported reciprocal relationship between Src and PRL phosphatases (25–27).

We therefore investigated if the regulation of SFK members by PRL-3 could be a mechanism mediating the signal from IL6 in MM.

Materials and Methods

We used the human myeloma cell lines INA-6, JN-3, and U266. INA-6 and JN-3 were kind gifts from Dr. M. Gramatzki (University of Erlangen-Nurnberg, Erlangen, Germany) and Dr. J. Ball (University of Birmingham, UK), respectively, and U266 was from ATCC. New cultures of cells were seeded at least every 4 months from vials aliquoted with cells propagated shortly after receiving the cells from their described original source, and they were regularly tested to ensure absence of mycoplasma. All cells were grown in RPMI-1640 supplemented with 2 mmol/L glutamine and 40 μg/mL gentamicin. INA-6 and JN-3 were grown with 10% and U266 with 15% heat-inactivated fetal calf serum (FCS). INA-6 is IL6 dependent and was cultured in media containing 1 ng/mL IL6. Cells were cultured at 37°C in a humidified atmosphere with 5% CO2. In order to deplete the cells of IL6 for experiments, cells were washed 4 times with Hanks’ balanced salt solution.

Antibodies, cytokines, and other reagents

IL6 was from Gibco (Invitrogen). Antibodies against Phospho-Src (Y416) (#2101), Phospho-Src (Tyro277; #2105), total Src (#2109), CSK (#4980), HCK (#14643), FYN (#4023), Phospho-Tyr- (P-Tyr)-1000 (#8954), Phospho-STAT3 (Tyr705; #9131), and total STAT3 (#9132) were from Cell Signaling Technology. The antibodies against PRL-3 (#318) and Mcl-1 (#8193) were from Santa Cruz Biotechnology, and the antibody against GAPDH (#ab8248) was from Abcam. PRL-3 inhibitor I (5-[4-Bromo-2-[(2-bromophenyl) methoxy] phenyl] methyl-2-thioxo-4-thiazolidinone) and SU6656 (2,3-Dihydro-N,N-dimethyl-2-oxo-[4,5,6,7-tetrahydro-1H-indol-2-yl]methyl-ene]-1H-indole-5-sulfonamide) were from Sigma-Aldrich, and PP2 Src inhibitor (4-Amino-3-(4-chlorophenyl)-1-(4-buty1)1H-pyrrozolo[3,4-d]pyrimidine, 4-Amino-5-(4-chlorophenyl)-7-(4-buty1)pyrazolo[3,4-d]pyrimidine) was from Santa Cruz Biotechnology. Gateway LR Clonase II Enzyme mix was from Invitrogen. PBMN-ires-GFP was a gift from Garry Nolan (Addgene plasmid # 1736), and plKO and shRNA-plKO against PRL-3 were a kind gift from Dr. Jim Lambert (University of Colorado, Denver, CO).

Lentiviral transduction for PRL-3 knockdown

293T packaging cells were transfected with either pLKO-shRNA against PRL-3 or pLKO (control plasmid) in combination with psPAX2 (packaging plasmids) and pMD2.G (envelope plasmid) for virus production. INA-6 cells were transduced with virions produced by packaging cells in order to establish INA-6 cells with knocked-down PRL-3 (shRNA PRL-3) and a control cell line (pLKO IN6).

Immunoblotting

Cells were treated as indicated and collected, pelleted, and homogenized in lysis buffer and immunoblotting method was performed as described previously (22). Images were acquired using LI-COR Image Studio Version 3.

Relative ATP measurement

CellTiter-Glo Luminescent (Ctg) Cell Viability Assay (Promega) was used to estimate the relative rate of cell viability by measuring the content of ATP present in the wells according to instructions provided by the manufacturer. In summary, cells were seeded in a 96-well plate, the provided assay reagent was then added to the plates, after which the plates were aged on a microplate shaker for 2 minutes, and kept at room temperature for 10 minutes before luminescence was determined. The luminescence signal was recorded with a VICTOR3 plate reader and Wallac 1420 Work Station software (PerkinElmer Inc.).

Luminex assay

Milliplex 8-plex Human SFK kit (Millipore #48-650MAG) was used to identify phosphorylated SFK members on the kinase domain (equals to Tyr416 in Src), including Src, YES, FYN, FGR,
LCK, HCK, BLK, and LYN, following manufacturer’s protocol. Briefly, lysates of cells were made by the lysis buffer supplied with the kit assay. Lysates were incubated with magnetic beads conjugated to selected phospho-SFK member antibody, and biotinylated antibody mixture was added. This was followed by addition of PE-conjugated streptavidin to quantify the level of active tyrosine phosphorylation of that SFK member (analogous to Tyr416 in Src). GAPDH beads (Millipore #46-667MAG) were added to adjust for protein load, in addition to measuring the protein concentration by the Bradford assay. Samples were read in a Bio-Plex 200 Systems (Bio-Rad Laboratories).

Statistical analysis
The statistical differences were determined by the Student t test using IBM SPSS Statistics 21.

Results
PRL-3–mediated survival of INA-6 is partially dependent on the catalytic domain
We have previously shown that overexpressed PRL-3 may in part execute the effects of IL6 and wanted to study in more detail signaling events regulated by PRL-3 in myeloma cells. From the IL6-dependent MM cell line INA-6, we generated cells expressing functional PRL-3 (PRL-3 INA-6), catalytically inactive PRL-3 (C104S INA-6), and empty vector control (Mock INA-6). PRL-3 overexpression was confirmed by both mRNA and protein level by quantitative real-time PCR (qRT-PCR) and Western blotting, respectively (Supplementary Fig. S1). Overexpression of PRL-3 both in catalytically active and inactive form significantly increased cell viability (Fig. 1A). However, cells with catalytically active PRL-3 were the most viable. As expected, the survival benefit of PRL-3 overexpression was more prominent in the absence of IL6, because IL6 induced PRL-3 expression also in Mock INA-6 (Fig. 1A; ref. 14).

We next investigated the influence of functional and catalytically inactive PRL-3 on the overall tyrosine phosphorylation pattern by using an antibody against P-Tyr in the presence and the absence of IL6. PRL-3 INA-6 in the absence of IL6 exhibited a tyrosine phosphorylation pattern reminiscent of that of cells grown in the presence of IL6, whereas C104S INA-6 and Mock INA-6 had distinctly different patterns in the absence of IL6 (Fig. 1B). These results confirm that PRL-3 is a key mediator of the IL6-driven signaling machinery and that ectopic PRL-3 makes the cells less dependent on IL6. The functional catalytic domain is necessary for this effect.

PRL-3 mediated survival through Src activation
Because several SFK members are activated in response to IL6 and are necessary for MM cell proliferation, the increased survival

Figure 1. PRL-3 increased viability and tyrosine phosphorylation profile of INA-6 myeloma cells. A, cell viability was measured in cells transduced by PRL-3 (PRL-3 INA-6), catalytically inactive PRL-3 (C104S INA-6), or control cells (Mock INA-6) by the CellTiter-Glo Assay. The mean of 6 independent experiments with 95% confidence intervals is shown. *, P < 0.05. B, cells were washed 4 times with Hanks’ balanced salt solution to deplete them of IL6, starved for 3 hours in serum-free medium, and cultured with or without 1 ng/mL IL6 for 5 hours. Global tyrosine phosphorylation profile was determined using phospho-Tyrosine (P-Tyr-1000) antibody. The membrane was re-probed with GAPDH as a loading control. Blot is one representative of three independent experiments.
and tyrosine phosphorylation could potentially be caused by Src activation (24).

As shown in Fig. 2A and B, PRL-3 increased Tyr416 phosphorylation of Src in the absence of IL6 in contrast to catalytically inactive PRL-3 and control vector. Tyr416 phosphorylation increased in the presence of 1 ng/mL IL6 in all cell variants. To elucidate mechanisms leading to Src activation, we investigated the level of C-terminal Src kinase (CSK), a known Src regulator, which suppresses Src activation by phosphorylation of Tyr527. We did not observe any significant differences among PRL-3 INA-6, C104S INA-6, and Mock INA-6 in Tyr527 phosphorylation or CSK level (Fig. 2C). Densitometry plots of Western blots in Fig. 2 are shown in Supplementary Fig. S2.

Next, we evaluated the effect of a small-molecule inhibitor of PRL-3 (PRL-3 inhibitor I) on Tyr416 phosphorylation in PRL-3–overexpressing INA-6 cells. As shown in Fig. 3A, PRL-3 inhibitor I in concentrations ranging from 10 μmol/L (half concentration of IC_{50}) to 40 μmol/L decreased active Src in both PRL-3 INA-6 and Mock INA-6 cells after 3- and 6-hour exposure. In order to see whether PRL-3 inhibitor I could also reverse IL6-induced Src activation, we exposed cells to ±1 ng/mL IL6 and 20 μmol/L PRL-3 inhibitor I. The inhibitor decreased IL6-induced Src activation, indicating that IL6 mediates Src activation via PRL-3 (Fig. 3B).

To confirm this result with an alternative method, we knocked down PRL-3 in INA-6 cells with shRNA and made stable cell lines with approximately 40% knockdown (shRNA PRL-3) and a mock vector control (pLKO INA-6; Supplementary Fig. 1C). Subsequently, we measured phosphorylation of Src in both shRNA INA-6 and pLKO INA-6 cells. As shown in Fig. 3C, shRNA PRL-3 cells in the absence of IL6 had less Tyr416 phosphorylation of Src than their mock counterpart, thus confirming our findings from the inhibitor experiments. However, in the presence of IL6, we observed no reduction of Src phosphorylation; arguably due to partial knock down efficiency, the cells still express enough PRL-3 for Src activation.

Finally, we wanted to investigate whether PRL-3 also influence Src activation in other MM cell lines. By treating the MM cell lines JJN3 and U266 with 40 μmol/L of PRL-3 inhibitor I, we observed a significant decrease in Src activation after 24 hours, showing that this was not exclusively found in INA-6 cells (Fig. 3D). Densitometry plots of Western blots in Fig. 3 are shown in Supplementary Fig. S3.

PRL-3 regulated activation of other SFK members

As we observed a prominent effect of PRL-3 on Src phosphorylation, we wished to investigate whether other SFK members such as LYN, FYN, and HCK could also be regulated by PRL-3. We confirmed expression of HCK, FYN, and LYN in...
INA-6 cells by qRT PCR (data not shown). We observed that HCK and FYN were upregulated approximately 10- and 2-fold, respectively, in C104S INA-6 cells compared with PRL-3 INA-6 and Mock INA-6. There was no significant difference in LYN expression between the three cell lines (Fig. 4A). We confirmed this on protein level by Western blotting (Fig. 4B). In order to evaluate the level of tyrosine phosphorylation within the kinase domain (analogous to Tyr416 in Src) in eight SFKs, we used the MILLIPLEX MAP8-plex Human SFK kit. PRL-3 INA-6, C104S INA-6, and Mock INA-6 had no FGR activity and very small or basal activity of BLK and LCK (data not shown). PRL-3 INA-6 had the same level of Src activity in both the absence and the presence of IL6, and the activation was significantly higher than in both Mock INA-6 and C104S INA-6, data which confirmed previous experiments. Cells expressing catalytically mutant PRL-3 showed even less Src activity than Mock INA-6 in both the presence and the absence of IL6 (Fig. 5A). Like for Src protein, C104S INA-6 cells showed the lowest level of phosphorylated LYN, and PRL-3 INA-6 had significantly higher LYN phosphorylation relative to both C104S INA-6 and Mock INA-6. Stimulation of cells with IL6 for 3 hours increased phosphorylation of LYN in C104S INA-6, but still C104S INA-6 showed significantly lower LYN activation than PRL-3 INA-6 and Mock INA-6 (Fig. 5B). Despite upregulation of total level of HCK and FYN in C104S INA-6 (Fig. 4), these cells did not show significant activation of the two IL6-dependent SFK members and had lower phosphorylated HCK than PRL-3 INA-6 in the absence of IL6 (Fig. 5C). Although not statistically significant, activation of FYN in the absence of IL6 followed a similar tendency as Src, LYN and HCK in transduced cell lines (Fig. 5D). Collectively, these results confirmed that PRL-3 could regulate both total amount and activation of several SFK members.

**Inhibitors of Src and PRL-3 reduced viability of cells overexpressing PRL-3, possibly mediated through Mcl-1 downregulation**

Finally, we examined whether cells with high expression of PRL-3 were responsive to two Src inhibitors, PP2 and SU6656. Increasing concentration of PP2 and SU6656 decreased the viability of PRL-3 INA-6 cells (Fig. 6A), which was accompanied by a reduction in the level of PRL-3 and the antiapoptotic protein Mcl-1, a known downstream target of Src (Fig. 6B). In order to examine whether a combination of PRL-3 inhibitor I and Src inhibitor had higher potential for decreasing viability of cells expressing high level of PRL-3, we used a very low concentration of the PRL-3 inhibitor (2 μmol/L) with both PP2 and SU6656. The low dose of the PRL-3 inhibitor I showed...
additional effect to both Src inhibitors (Fig. 6A). To explore the mechanism of PRL-3 and Src inhibition, we measured the effects of both PRL-3 inhibitor I and PP2 on PRL-3 and Mcl-1 expression. Each inhibitor given separately, at low concentration, had no effect on the PRL-3 level, but caused a small reduction of the Mcl-1 level. However, using both inhibitors at the same time decreased the level of both PRL-3 and Mcl-1 (Fig. 6C). Collectively, the results show that combining PRL-3 and Src inhibitors could reduce the beneficial effect of PRL-3 on viability. We have previously shown that PRL-3 contributes to IL6-mediated activation of STAT3 in MM cells (22). To exclude the possibility that Src was an intermediate between PRL-3 and STAT3, we measured STAT3 activation after Src inhibition. While the Src activation was reduced, we did not see any change in the STAT3 phosphorylation level (Fig. 6D). Densitometry plots of Western blots in Fig. 6 are shown in Supplementary Fig. S4.

**Discussion**

A number of studies show the association between elevated PRL-3 expression and the development of solid tumors and hematologic cancers, suggesting that this phosphatase could be a good target for treatment (21). We have previously shown that IL6 increases the expression of PRL-3 in MM cells, and that this phosphatase to some degree can replace the IL6 effect on survival (14, 22). In the current study, we have explored the oncogenic roles of PRL-3 in MM in more detail and found SFK members as mediators of PRL-3 effects. We show that PRL-3 regulated expression and activation of Src, LYN, HCK, and FYN, four important IL6-dependent oncogenic SFK members (24).

Expression of functional PRL-3 increased phosphorylation of Src on Tyr416, which could be a signaling event contributing to increased survival in cells expressing functional PRL-3. Conversely, inhibition of PRL-3 decreased this phosphorylation, confirming the role for PRL-3 in Src activation. Our results are consistent with other studies showing that ectopic expression of PRL-3 promotes proliferation and migration by activating Src kinase (26, 28). However, the mechanism for Src activation we found in this study was different from what has been reported for Src activation by PRL-3 previously (28, 29).

We found that PRL-3 caused Src activation by increasing phosphorylation of Tyr416, but had no effect on the CSK level or on phosphorylation of Tyr527 in Src. In a study done on HER293 cells, PRL-3 expression caused a reduction in the CSK level, leading to Src activation by decreasing phosphorylation on Tyr527 (28, 29). They could not find any significant change in phosphorylation on Tyr416 in cells overexpressing PRL-3. However, this study is in accordance with a previous report that PRL-3 is important for VEGF-induced phosphorylation of Src on Tyr416 and increases the migratory and invasive properties of endothelial cells (26).

The importance of Src in MM cells has been highlighted by others. For instance, in one study, overexpression of phosphorylated Src in RPMI8226 MM cells made them resistant to different anticancer drugs, such as adriamycin, vincristine, dexamethasone, and melphalan (30). In another study, constitutive autophosphorylation of Src at Tyr416 was shown to be important for survival and proliferation of patient-derived MM cells, which indicates that Src activation in MM is of clinical relevance (31). Recently, Src inhibition was introduced for the treatment of MM-associated osteolytic bone disease, which is one of the main causes of morbidity in MM (32).

Other SFK members than Src, like LYN and HCK, are primarily found in hematopoietic cells and are also important...
However, they have not been studied in the context of PRL-3 and MM pathophysiology. When we measured the activation of eight different SFK members, we observed that not only Src but also LYN and HCK had significantly higher activation level in PRL-3–overexpressing cells in the absence of IL6. Although the total amount of HCK and FYN was higher in C104S INA-6, they did not show a higher activation level in C104S INA-6 compared with functional PRL-3–overexpressing cells. In the myeloma cell line U266, activation of STAT3 and ERK1/2 is not sufficient for proliferation in response to IL6; LYN activation is also needed. LYN activation is dependent on association of LYN with CD45 tyrosine phosphatase upon IL6 stimulation (11, 34, 35). Similarly to Src and LYN, HCK mediates proliferative and survival effects of IL6 by binding to IL6ST and phosphorylation of GAB1 and GAB2 docking proteins in MM cells (36). Therefore, increasing activation of Src, LYN, and HCK by PRL-3 supports the oncogenic properties of PRL-3 in MM. Increased total amount of HCK and FYN in cells expressing catalytically inactive PRL-3 could be the result of diminished negative feedback regulation of SFK members by their active forms. Previous studies with knockout of individual SFK members led to rather subtle phenotypes suggesting functional compensation by other family members. This is probably the consequence of vital roles of SFK members in cells (37, 38).

The observation that C104S INA-6 had an even lower activation level of several SFK members than Mock INA-6 could be explained by a dominant-negative effect blocking residual active PRL-3 in C104S INA-6. However, we did not see any dominant-negative effect on cell survival. To the contrary, C104S INA-6 had a reduced level of apoptosis as compared with Mock INA-6. One possibility could be that PRL-3 has some other domain than the catalytic domain that is important in regulation of survival, which needs further investigation.

To sum up our previous (14, 22) and current findings on PRL-3, we suggest that PRL-3 is an effector protein downstream of IL6, that it induces activation of STAT3 and SFK members and creates a positive feedback loop in both signaling pathways. However, the possibility of activation of STAT3 through SFK was not confirmed, as using inhibitor of Src did not affect STAT3 phosphorylation. Subsequently, the Mcl-1 level increases downstream of both Src and STAT3 and leads to enhanced cell survival (Fig. 7).

Despite the large number of studies showing a link between PRL-3 overexpression and poor prognosis in various cancer types, less is known about signaling pathways governed by this phosphatase. Our results add valuable insights into the signaling mechanisms regulated by PRL-3 in MM cells with deregulation of SFK family members LYN, Src, HCK, and FYN and increased level of Mcl-1. Our study points
to the importance of both Src and PRL-3 in MM pathology, and to Src and PRL-3 as potential targets for treatment of multiple myeloma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Figure 6.
Inhibitors of Src and PRL-3 reduced viability of cells overexpressing PRL-3 by downregulating Mcl-1. PRL3 INA-6 were depleted of IL6 and (A) exposed to increasing concentrations of Src inhibitors PP2 and SU665 with or without 2 μmol/L PRL-3 inhibitor (B) overnight and viability was measured by the CellTiter-Glo Assay. The figure shows one representative of three independent experiments. Error bars represent ± SD of triplicate measurements. B, cells were cultured with 6.5 μmol/L PP2 overnight. The same membrane was re-probed for PRL-3 and GAPDH. C, cells were cultured overnight with PRL-3 and Src inhibitor PP2 alone or combined. Mcl-1 and PRL-3 levels were measured by immunoblotting. Membranes were re-probed for GAPDH. D, cells were starved for 3 hours in serum-free medium before treatment with 6.5 μmol/L PP2 and 1 ng/mL IL6 for 3 hours. The same membrane was re-probed for total Src, pSTAT3, total STAT3, and GAPDH. Blots are one representative of three independent experiments.

Figure 7.
Proposed mechanism for cancer progression by PRL-3 in MM. PRL-3 is important downstream of IL6 in induction of expression (open arrowheads) or activation (solid arrowheads) of signaling molecules and the antiapoptotic protein Mcl-1.
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